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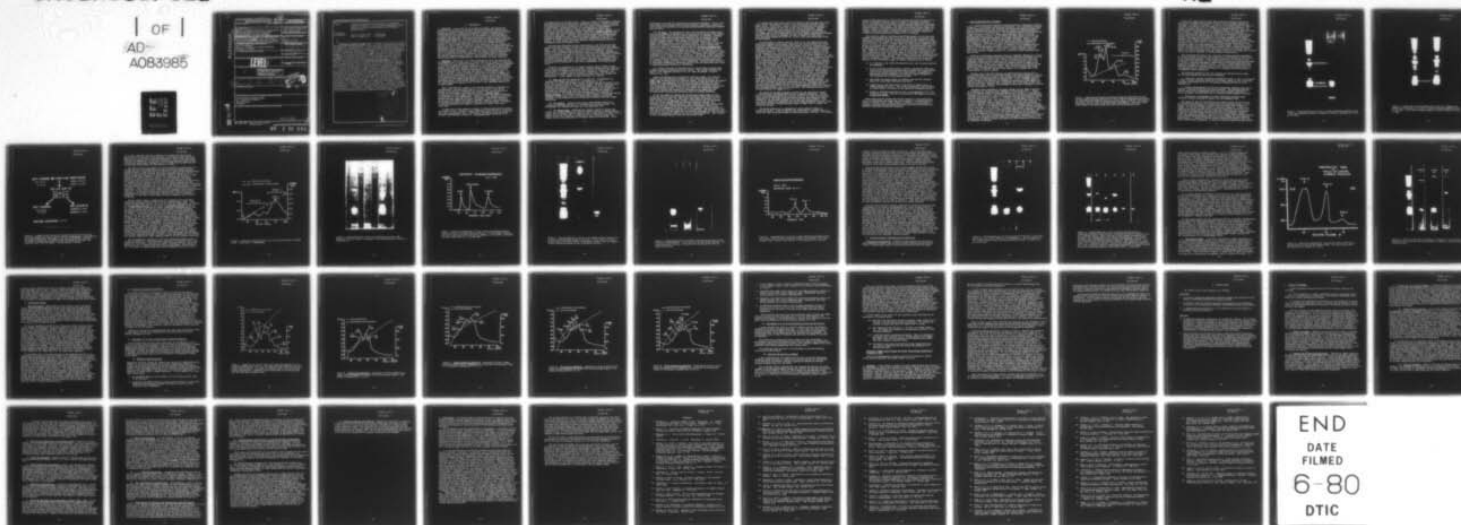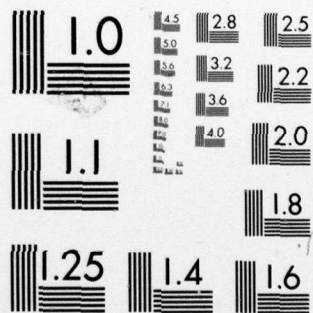   JUL 79   P R OELTGEN, J K OELTGEN   N00014-76-C-0485
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Project: Isolation and Chemical Characterization of Hibernation
            Induction "Trigger(s)" in the Plasma of Hibernating Ground
            Squirrels (Citellus tridecemlineatus) and Woodchucks
            (Marmota Monax).

Co-Principle
Investigators: Peter R. Oeltgen, Ph.D.   035-28-1444
                  Jean K. Oeltgen, M.S.   306-44-3813

A hibernation induction trigger (HIT) is present in the plasma of hibernating woodchucks and ground squirrels which can induce hibernation when injected in summer-active ground squirrels. Recent biochemical characterization of this HIT molecule in our laboratory utilizing three distinct resolving techniques, isoelectric focusing, preparative isotachophoresis, and affinity chromatography clearly indicates that this molecule is bound to or closely associated with albumin in the plasma of hibernating animals. Moreover, our recent efforts to develop an assay for HIT activity of resolved plasma fractions utilizing primates as test animals has given the first indication that this molecule may initiate transient yet profound physiological alterations in these animals. When small amounts of the HIT-active plasma fractions are infused into the brain ventricle fluid of monkeys we have noted some truly remarkable physiological responses such as depressed metabolism, hypothermia, decreased heart rate and the appearance in some animals of an anesthetized state.

Our goals are two-fold, we intend to isolate a completely homogeneous HIT molecule which is dissociated from albumin utilizing the techniques of preparative gel electrophoresis and column chromatography. Once this short-term objective has been achieved, we will estimate the molecular weight of the HIT molecule by SDS-polyacryla gel electrophoresis and analytical ultracentrifugation and identify the site of production of the molecule in hibernators utilizing immunofluorescent techniques. Our long term biochemical characterization goal is to determine the amino acid composition and sequence of the HIT molecule and ultimately to synthesize it chemically. A second, an equally important goal is to fully establish a bioassay for the HIT molecule utilizing non-hibernators. In so doing, we intend to fully explore the clinical potential of the molecule in such areas as cryosurgery, organ preservation, tumor inhibition and anesthesiology through collaborative research efforts.

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A. INTRODUCTION

1. OBJECTIVES. A major goal of our research activities, both present and future, is to isolate and to eventually completely chemically characterize a hibernation induction trigger(s) (HIT) from the plasma of hibernating woodchucks (Marmota Monax) and 13-lined ground squirrels (Citellus tridecemlineatus). A secondary goal has been directed at the development of a rapid and specific in vitro or in vivo assay utilizing non-hibernators to monitor HIT activity of resolved plasma fractions. The latter goal seems very near at hand as indicated by preliminary studies in one of our laboratories and through collaborative studies with several other laboratories utilizing our resolved HIT-active fractions. These studies have given the first indication that the isolated HIT molecule may elicit intense reversible biological responses (depressed metabolism, hypothermia, decreased heart rate, an anesthetized state and qualitative and quantitative enzyme alterations) in primates and other nonhibernating recipients. Thus it is becoming increasingly clear that the complete chemical identification and localization of the site of production of this blood borne HIT molecule may have far ranging clinical impact in such divergent fields as cryobiology, organ preservation and transplantation, anesthesiology and tumor inhibition.

2. BACKGROUND. Hibernation is an unique phenomenon in which the entire animal participates; that is, each organ at a tissue level may be capable of hibernating. It has been most aptly defined by Hoffman (1) as "a regulated, periodic phenomenon in which body temperature becomes readjusted to new, lower levels approximating ambient, and heart rate, metabolic rate and other physiological functions show corresponding reductions from which spontaneous or induced arousal to normal levels is possible at all times with the addition of environmental heat." It is one of the most striking circannual rhythmicities to be seen in mammals and is most precisely demonstrated in the rodential ground squirrel and woodchuck. These animals possess specialized regulatory mechanisms which annually acclimize or prepare them for impending hypothermia and ultimately permit them to enter the hibernating state in which energy expenditure and food consumption are minimal. Yet winter hibernation per se is only one phase in the remarkable annual cycle of these animals.

Thus, a more wholistic concept of the seasonal hibernator is obtained with consideration of the three major phases of its life cycle. These may be defined as the activity, hibernation and arousal states and are explained as follows with subordinate classifications included. Activity state-- In spring (and in early summer) after terminal arousal from winter hibernation, the hibernator is truly homeothermic. During this time, reproductive activities dominate and most endocrine activities are maximal. Hibernation induction--- At the end of the summer (late August, September) appetite, basal metabolism and spontaneous activities decline and endocrine secretions change. Presumably, if internal preparations are complete, hibernation is entered into from a sleep state.

It takes place in the presence or absence of food and water when the animals are exposed to cold. Declines in body temperature follow declines in respiration, heart rate, and metabolic rate. Consequently, hibernation is not due simply to deranged thermoregulatory mechanisms. Hibernation state--Hibernation is not

a prolonged period of constant torpor. Periods or "bouts" of hibernation regularly alternate with periods of arousal and homeothermic existence. Periodic arousings are characteristic of all hibernators, but their cause of physiological necessity is unexplained. Arousing may be due to an accumulation of some metabolic product. In the short space of 2 hours, body temperatures, heart rates, respiration, and heat production increase dramatically. Terminal arousing--In the late winter-early spring (February, March) subtle but definite changes in blood and tissues take place. Gradually, some endocrine glands acquire morphological evidence of renewed activity, reproductive organs develop, and the "bouts" of hibernation shorten in duration with consequent lengthenings in periods of arousal. In early spring, the animal terminally arouses and enters the active homeothermic state and breeding occurs.

Data have been accumulated by many investigators on the function and regulation of systems relating to various tissues of hibernators in all three major phases. Yet, little is known of the actual mechanism of induction into the hibernation state, involving changes from high to very low metabolism. Lending support to the hypothesis that hibernation induction is more than themoregulated are such researchers as Lyman (2) who states "that onset of hibernation in Citellus tridecemlineatus is presaged by a decline in heart rate which always precedes the decline in body temperature. This suggests that the heart is being actively slowed by something other than a reduction in temperature, and emphasizes that the entrance into hibernation is more than an abandonment of the warm-blooded state."

Therefore, the suggestion of a "trigger" substance which actively induces entry into the hibernation state has gained sizeable acceptance and has implicated a large number of endogenous substances from a variety of tissues--all cited for their ability to trigger, induce, or maintain hibernation. Included in this group are such diverse tissues and molecules as brain, brown fat, hormones and electrolytes. A brief summary of these researches is as follows:

(a) Brown fat has been implicated for a long time. See Rasmussen (3); Johansson (4); Goldman and Bigelow (5); and Bigelow et al. (6). wrote that "an active humoral agent related to hibernation may be present in the blood, and probably brown fat of hibernating animals. We may be searching for an extremely labile substance, or a very minute amount of an active substance, which would be difficult to extract or identify." However, its presumed role as an inducer of hibernation has certainly switched following the research of Smith and Hock (7) who showed that brown fat (per se) is (or contains) an arousal substance, but evidently cannot be shown to contain a hibernating inducing substance! Despite this, brown fat remained until recently as a possible contender for the production of other blood-borne substances which may trigger hibernation.

(b) The pancreas. Insulin also has had a long-standing reputation as a hibernation-inducing substance (if introduced into the blood stream). See Laufenberger (8); Dworking and Finney (9); and Suomalainen (10).

(c) The adrenal gland. Following upon original research by Popovic and Vidovic (11) in which hibernation could be induced in season in adrenalectomized animals (which otherwise would not hibernate) by a simple graft of a small piece of adrenal cortex into the anterior chamber of the eye of such an animal, the adrenal had also been implicated as a hormonal source for hibernation. Kayser

-2-

and Petrovic's research (12) extended this observation remarkably. There is now little doubt that a careful balance of known hormonal secretions is required in order to have any hibernator in a condition wherein possible induction of hibernation can occur.

(d) The brain. The contention by Kroll (13) and Axelrod (14) which states that a brain substance (presumably not circulated) can be extracted which induces recipient animals, which are not normally hibernators, to hibernate. Although not directly implicated in hibernation, Monniers (15) has obtained a sleep inducing substance (a dialyzable) factor obtained from the cerebral venous blood of sleeping rabbits. This substance (a peptide having a M.W. 700) is capable of inducing sleep in active recipient rabbits. More recently, Swan and Schätte (16) prepared a partially purified polypeptide extract derived from subcortical brains of hibernating ground squirrels, Citellus tridecemlineatus. Aliquots of this extract were infused into rats with an inlying cephalic vena caval catheter at a dose of 300 mg/kg body weight. The slow infusion into rats of this extract over a fifteen minute period resulted in a mean decrease in oxygen consumption of 65 percent of control values at 30 minutes and a decline in body temperature of 5° C within one hour of infusion. The decline in body temperature to 31.7° C without evidence of shivering persisted from 75 minutes to 30 hours. The infusion of brain extracts of non-hibernating ground squirrels resulted in no significant changes in either of these parameters. Swan and Schätte referred to extractable metabolically depressant agent(s) from the subcortical brain tissue of hibernating ground squirrels as "antabolone."

(e) Finally, four ions in the blood have at various times and places been studied as possible hibernation-affective agents. They are Mg++ (Riedesel and Folk,[17]); Na+ (Pengelley and Kelly, 18); Ca++ (Myer and Sharpe, 19) and K+ (Willis et al., 20).

Despite the multiplicity of hibernation induction studies, the literature provides scant experimentation designed to truly induce hibernation in the summer time, and hence break the circannual rhythm. Unlike our current and planned studies, most experimentation in which the above-named substances had been introduced to induce hibernation were performed during the hibernation season. Thus, propensities for hibernation were set against the circannual backdrop which favored hibernation anyway. Our major assay method for blood-borne trigger until quite recently was induction of hibernation in the summer when the recipient ground squirrels and woodchucks would not normally hibernate.

Of the vast number of chemical and physiological changes known to occur during the states of activity, hibernation and arousal, blood related studies provide much of the data. In a year round study, Dawe and Spurrier (21), in 1968, reported on the hematologic changes in the blood of the 13-lined ground squirrel, and the expanded data were published (Spurrier and Dawe, 22). Differences were observed in hematocrit, mean corpuscular volume, osmotic fragility of erythrocytes, folding and flattening of erythrocytes, lack of cold agglutinins or aggregation of cells, white cells, platelets and the presence of large crystalline and lipid particles in the circulation. Dawe and Spurrier feel that some or all of these changes may have definite advantages for free circulation of blood and exchange of oxygen to the tissues during hibernation. These changes follow a circannual rhythm in tune with the various states.

In another year round study, Galster and Morrison (23) have shown that blood lipid, protein and hematocrit levels in the 13-lined ground squirrel follow yearly cycles. Total lipid, α-lipid, β-lipid and chylomicrons were minimal in late spring and maximal in late fall at the onset of hibernation. Moreover, proteins were minimal in late spring except γ-globulin which was minimal in January. A most interesting finding by these researchers and one which we will expand upon in work accomplished is that serum albumin concentration increased threefold (from 18 g/l) by midsummer and then to fourfold in early fall, a level maintained through the winter (av. 65 g/l) until early spring when levels decrease sharply.

In blood studies of shorter duration, Biorck et al. (24) demonstrated that hibernating hedgehogs had prolonged coagulation and recalcification time during hibernation compared with non-hibernating animals and that no circulating anti-coagulants were demonstrable and Denyes and Carter (25) noted extremely prolonged blood-clotting in hibernating hamsters which is due to a combination of reduced prothrombin synthesis. The latter result is probably due to a general lowering of body metabolism and a high concentration of some heparinoid substance in the plasma. Antibody production during hibernation is also varied. Beudoin et al. (26) showed that antibody production to helminths still occurred in hibernating woodchucks while Larsen (27) found that the half-life of IgG_2 was prolonged from 2.2 to 40 days in hibernating animals. Moreover, the survival of erythrocytes is affected in the hibernating state. The life span of erythrocytes in hibernating hamsters was 160 days according to Brock (28) which is over double that of controls (78.5 days). This is either due to decreased erythrocyte destruction or to lessened amounts of erythrocyte formation. Brock also noted decreased ATP levels in erythrocytes of hibernators and Bito and Roberts (29) demonstrated that plasma glucose and lactate levels were significantly decreased in hibernators. Such findings necessarily indicate that less energy is available to the cells in hibernating animals. The affinity of hemoglobin for oxygen is also significantly altered in hibernating animals. Our own isolectric focusing studies (30) utilizing hemoglobin from ground squirrels in their various activity states indicate major quantitative and qualitative alterations in the hemoglobin molecule. This will also be detailed in the work accomplished section. Burlington and Whitten (31) and Larkin (32) demonstrated that hibernation blood of ground squirrels contained significantly less 2,3-DPG, even though hemoglobin content was unaltered. Their findings provide a basis for the hypothesis that hibernating animals have increased affinity for oxygen at 37° C.

The reason for the much prolonged survivability and contractile strength of isolated hearts from hibernators may have a chemical explanation. Calcium levels in hibernating hamsters were found by Ferren et al. (33) to be increased significantly in the serum, heart and skeletal muscle, while magnesium levels increased in the serum but decreased in the heart and skeletal muscle. Their observations support the hypothesis that calcium might accumulate in muscle tissue (especially the heart) during hibernation where it might serve to enhance the contractile process.

The arousal process too is accompanied by marked chemical changes in hibernating animals. Klain and Whitten (34) noted markedly increased levels in plasma free amino acids upon arousal of hibernating ground squirrels. The probable

-4-

origin of these amino acids is from tissue proteins and their role might be to provide an immediate substrate source in support of thermogenic processes. This is consistent with the finding of increased glucose production from amino acids during the arousal period. The markedly elevated levels of creatine and creatinine noted by Kristofferson (35) upon spring arousal further support this concept. However, the adaptive significance and the biochemical initiator(s) leading to this wide variety of chemical changes in the tissues resulting in a number of altered physiological responses still is unknown.

Since both the chemical constituents and formed elements of the blood were markedly altered during the states of activity, hibernation, and arousal, Dawe and Spurrier (1968-1973) began to speculate whether the blood might not contain substances which play a major role in regulating mechanisms of hibernation induction or activity. In 1966, a "trigger" which can induce natural mammalian hibernation was discovered to be present in the blood of hibernating ground squirrels by Dawe and Spurrier (36). Somewhat more specific characterizations of the blood trigger for hibernation have been reported by Dawe and Spurrier. (37) since then. From 1968 to 1975, the "trigger" has been successful in inducing hibernation in hundreds of animals during the summer (a season when these animals do not ordinarily hibernate). Non-transfused animals (controls) are maintained under similar conditions as experimentals. This is further proof that the hibernation cycle is circannual in nature. The preliminary characterization by Dawe and Spurrier of this blood-borne trigger for natural hibernation are as follows.

a) It is present in the serum of hibernation blood, both in ground squirrels and woodchucks.

b) It acts intra-and interspecifically insofar as appropriate hibernation serum and 13-lined ground squirrels when transfused can trigger hibernation in other ground squirrels of the same species, and appropriate hibernation serum of woodchucks can similarly trigger hibernation in both woodchucks and 13-lined ground squirrels.

c) The trigger can induce animals to hibernate even when the recipient remains in a warm-light room during the winter.

d) Trigger has not been found either in the blood of summer active, or arousing animals in winter. Thus, effective material is not described as "winter blood," but rather, by the expression "hibernation blood."

e) Trigger is inactivated partially at 20° C, and completely at 37° C for 30 minutes in vitro. This will be detailed more thoroughly in the work accomplished section.

While these previous studies have focused primarily on the physiological effects of hibernation and trigger the area of chemical characterization and specific isolation as well as any attempts to develop a rapid and specific in vitro or in vivo assay preferrably utilizing non-hibernators has been left until our recent work essentially unexplored.

3. WORK ACCOMPLISHED AND IN PROGRESS

a. Isolation and Partial Chemical Characterization of a Hibernation Induction "Trigger(s)" in the Plasma of Hibernating Woodchucks. Prior experimental observations of Dawe and Spurrier (38) indicated the hibernation induction trigger (HIT) could be completely inactivated by warming the plasma from hibernating ground squirrels to 37° C for one-half hour. The thermolabile nature of this blood-borne trigger indicated to one of the investigators (Oeltgen) that it was either peptide or protein in nature. Such an observation also tended to rule out the prostaglandins as possible candidates for trigger molecule(s) since it is well-documented that these are relatively thermostable molecules. However, three major classes of molecules or ions still remained as possible contenders for the "trigger(s)". These were the lipids and phospholipids which have been shown by Bragdon (39) to be the highest in hibernators of any mammalian species assayed, the catecholamines, especially those of brain origin and small ions such as Na^+, K^+, Ca^{++} and Mg^{++}. These last two groups have been virtually ruled out as possible trigger molecules by a relatively simple desalting experiment in 1976.

Thirty ml of hibernating woodchuck plasma was completely desalted in a 2 hour period by utilizing a hollow fiber devise (Bio-Rad, Bio-Fiber 50-Beaker) which has a molecular weight cut-off of 5,000. Hence, all molecules of M.W. 5,000 and lyophilized residue was then assayed for HIT activity by Spurrier then in the department of Physiology at Loyola University Medical Center in summer-active ground squirrels. In all cases, animals which had received this desalted plasma preparation hibernated within two weeks. These results indicated the trigger was in excess of 5,000 M.W. and gave added support to the concept that it was either protein or lipid in nature.

The next step in the 1976 isolation and characterization protocol was to electrophoretically resolve the desalted plasma by the highly sensitive resolution technique of isoelectric focusing. The technique has been thoroughly described by Svenson (40), Vesterberg (41) and Haglund (42). It is a procedure which is ideally suited for the analytical or preparative separation of mixed ampholytes, especially proteins, having as small a difference as 0.02 pH units at their isoelectric point (pI). Moreover, the technique is often used to determine a physical constant of a protein, its pI.

Throughout these experimental procedures extreme care was taken to maintain the plasma and all resolved protein components of the plasma at 4° C. Twenty mg of the desalted plasma was electrofocused for 48 hr at 500 V in a pH gradient extending from 3.5 to 10.0. The results of this most interesting electrofocusing experiment are depicted in Figure 1. Plasma was resolved into 5 distinct protein fractions having pIs of 4.5, 5.2, 5.5, 6.3 and 7.0 as determined from the plot of the increasing pH curve. This procedure was carried out 4 more times in order to obtain sufficient resolved sample for bioassay. In each case, the highly sensitive isoelectric focusing technique resulted in an almost identical elution profile at 280 nm. In no instance did the pI of the 5 resolved protein components vary by more than 0.1 of a pH unit. To expedite the bioassay, the protein components having pI values of 4.5 and 5.2 were pooled and referred to as Fraction I, while the protein component having a pI of 5.5 was termed Fraction II and the remaining two protein components having pIs of 6.3 and 7.0 were pooled and represented Fraction III. Any lipid or phospholipid components would necessarily have migrated into the anode electrode solution (comprised of phosphoric acid) because of the preponderance of negative charges associated with these molecules and should thus be completely inactivated.
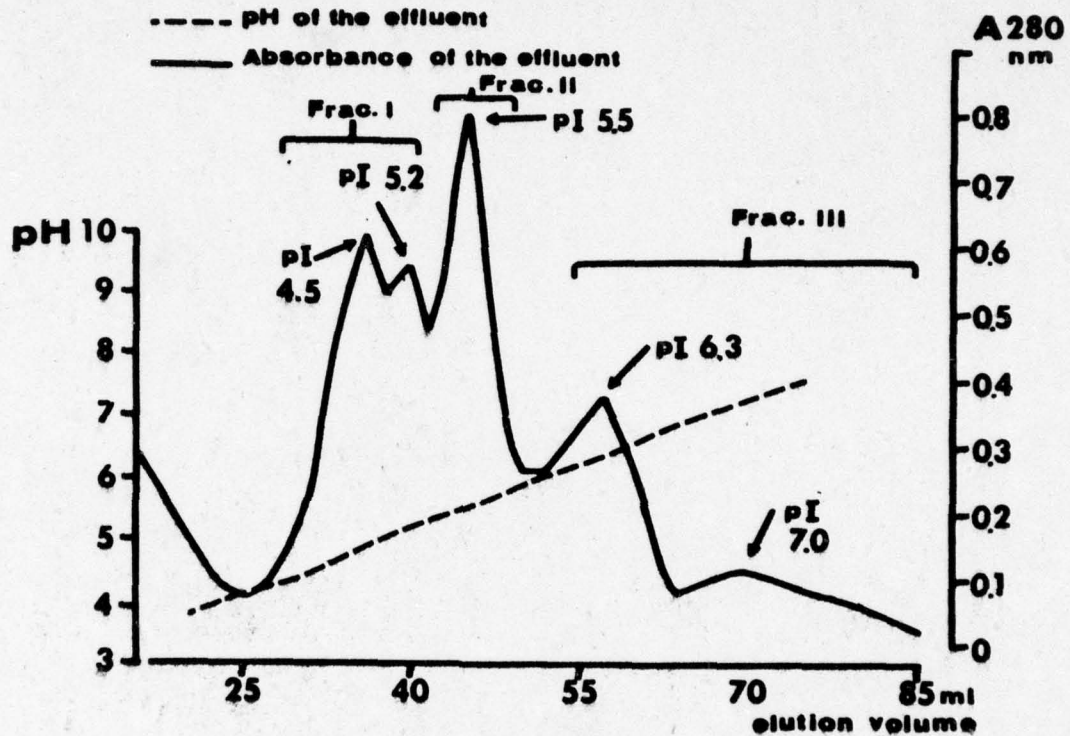
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Figure 1. Separation of hibernating woodchuck plasma by isoelectric focusing in a pH gradient extending from 3.5-10.0 using a LKB 8100 Electrofocusing Column. The curve with peaks shows the absorption of the eluate at 280 nm. The steadily increasing curve is a plot of the pH gradient superimposed. Isoelectric point values shown at the various peaks were read from the pH gradient curve.

These three plasma fractions were then assayed for biological activity in three groups of summer-active ground squirrels, each group being comprised of 10 animals. Aliquots of Fractions I, II and III, at a concentration of 3 mg/ml of 0.9% NaCl, were injected into the saphenous vein of the ground squirrels. All animals receiving Fractions II or III failed to hibernate whereas 8 out of 10 ground squirrels receiving Fraction I hibernated within 2 to 6 days after receiving the injection. Polyacrylamide gel electrophoresis of 400 μg of Fraction I (Fig 2) indicated that albumin was the chief protein constituent of this fraction and the major protein peak of Fraction I had a pI of 4.5 which is the physical constant previously well-established for the albumin component of the plasma. Moreover, this finding correlated well with the findings of Galster and Morrison (23) who noted a 3-fold increase in serum albumin by late summer rising to a 4-fold increase in early fall (Fig 3), a level maintained through the winter and then decreasing sharply in spring immediately following arousal. Such shifts in serum albumin correspond well with the different activity states of these hibernators. Hence, a distinct possibility exists that the HIT activity of Fraction I may be bound to or closely associated with the albumin fraction of the plasma and its physiological role may be dependent on changing albumin concentrations.

This constituted the brunt of our isolation and characterization experiments as of 1976, keeping in mind the time restrictions of the bioassay for HIT activity of resolved plasma fractions. Since albumin is noted for its ability to bind a wide variety of molecules, our 1977 experimental protocol was centered on attempts to isolate a completely homogeneous albumin fraction from hibernating woodchuck plasma and to assay this for HIT activity. In order to achieve this goal, three different experimental techniques were utilized, each falling slightly short of our original goal, yet further motivating us to ultimately achieve resolution of a homogeneous albumin preparation.

The techniques employed in the 1977 isolation and characterization scheme for HIT active plasma fractions were the following:

a. Isoelectric focusing of hibernating woodchuck plasma in a pH 3.5-10 gradient in order to obtain sufficient quantities of Fraction I which, in turn, could then be further fractionated by IEF in a pH gradient extending from pH 3.5 to 6.0 (Haglund, 42).

b. Affinity Chromatography of both whole hibernating woodchuck plasma and of Fraction I obtained by the electrofocusing technique. The technique utilizing Affi-Gel Blue as the chromatography matrix has been thoroughly described by Travis and Dannell (43) and Wille (44) to selectively absorb albumin from serum.

c. Preparative isotachophoresis of whole hibernating woodchuck plasma following a protocol described by Haglund (45) and Hjalmasson (46).

The results of the 1977 fractionation and bioassay results for HIT activity of resolved plasma fractions are as follows and gave additional support to the concept that the HIT molecule is indeed closely associated with or bound to albumin. An overview of these results is depicted in Figure 4. A total of 40 summer-active ground squirrels were injected and assayed for HIT activity with fractionated preparations by the three previously cited separation techniques. A total of 18 of these summer-active ground squirrels hibernated. However, a much more impressive figure is that 16 out of 21 animals hibernated when injected with resolved hibernating plasma fractions in which albumin was the predominant plasma protein. A total of 8 control animals received physiological saline and none of these animals hibernated. A total of 8 different plasma fractions derived
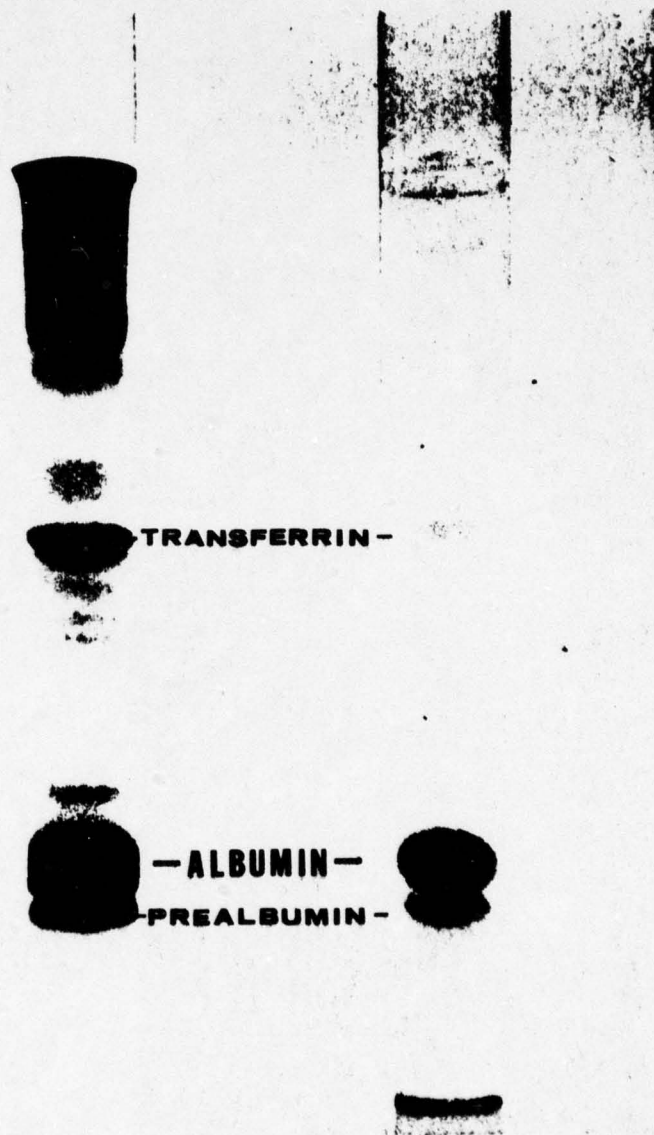
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Figure 2. Polyacrylamide gels of 400 µg of whole hibernating woodchuck plasma on the left compared to Fraction I derived by Isoelectric focusing, pH 3.5-10, on the right.

Figure 3. A comparison of polyacrylamide gels of 400 μg of summer-active woodchuck plasma on the left to that of winter-hibernating woodchuck plasma on the right. A much denser albumin component is clearly visible on the gel at right.

AFF. CHROM. ← **HWP** → **ISOTACHO.**

NA (1:4)   FRAC 5 (0:6)

A (3:4)   FRAC 7 (4:6)

IEF pH 3.5–10

FRAC I (8:10)

FRAC II (0:10)

FRAC III (0:10)

AFF. CHROM.   **IEF pH 3.5–6**

NA (1:6)   SUBFRAC B (0:3)

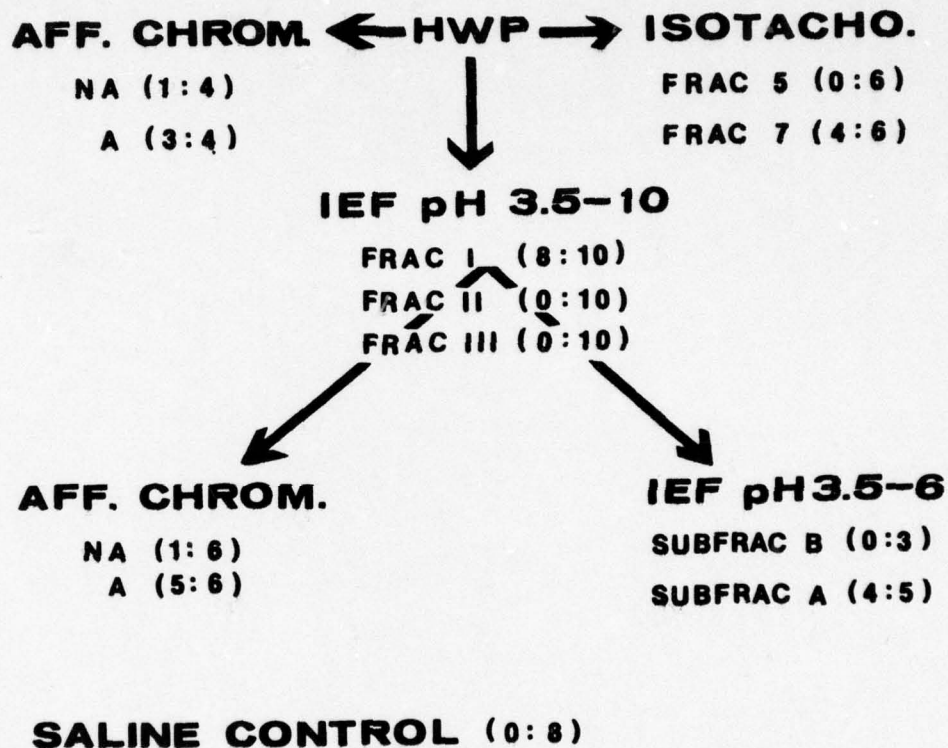A (5:6)   SUBFRAC A (4:5)

SALINE CONTROL (0:8)

Figure 4. A summary of our 1976 and 1977 bioassay results for HIT activity of resolved plasma fractions derived by Affinity Chromatography, Isotachophoresis and Isoelectricfocusing. The number on the left in parenthesis, indicates the number of animals which hibernated while the number on the right indicates the size of the test groups. (NA-non-albumin; A-albumin)

-11-

by the three different isolation procedures to be assayed for HIT activity
were given to W.A. Spurrier of the Department of Physiology, Loyola University .
Medical Center without any accompanying information. She, in turn, gave these
fractions an alphabetical listing from A through H and injected 3 mg/ml of each
fraction into appropriately segregated groups of summer-active ground squirrels
and monitored these animals for hibernation in the cold.

The results of these experiments will now be presented in greater detail to
document the role that albumin plays in resolved HIT active plasma fractions.
A pooled Fraction I sample derived from 8 IEF runs at pH 3.5-10 was subfractionated
further by IEF utilizing a narrower pH gradient extending from pH 3.5 to 6.0.
This electrophoretic resolution is indicated in Figure 5. The pH curve is super-
imposed on the elution profile at 280 nm. The electrophoretic resolution
resulted in a major protein peak referred to as Sub-Fraction A and several
minor peaks which were pooled for bioassay purposes and referred to as Sub-Fraction
B. Bioassay results indicated that zero out of 3 animals injected with Sub-
Fraction B hibernated whereas 4 out of 5 animals receiving Sub-Fraction A hibernated.
Polyacrylamide gel electrophoresis of 400 µg of these resolved fractions is depicted
in Figure 6. Sub-Fraction A on the right is compared to Sub-Fraction B in the
Center and, in turn, both of these subfractions are compared to the starting material,
Fraction I. One can easily perceive that the HIT-active Sub-Fraction A has a
preponderance of albumin and prealbumin components and, while not completely
homogeneous, it does have a lesser number of protein components than Fraction I
on the left.

The next figure (Figure 7) demonstrates the resolution of 45 mg of whole
hibernating woodchuck plasma through an affinity chromatography column. Three
distinct elution peaks resulted. The first is simply the column wash. The second
is the non-albumin fraction, and the third is the albumin fraction. Three out
of 4 ground squirrels receiving the albumin preparation hibernated whereas only
one out of 4 animals which had received the non-albumin fraction hibernated.
The homogeneity of these two fractions is depicted by the polyacrylamide gels
in Figure 8. On the left is whole plasma from hibernating woodchuck; the center
gel is that of the non-albumin fraction, while that on the right is the albumin
fraction. One can note that even the non-albumin fraction is not completely free
of albumin and its quite possible that this albumin contamination probably accounted
for the hibernation in 1 out of 4 animals assayed for HIT activity with the non-
albumin fraction. Affinity chromatography of 55 mg of Fraction I from IEF, pH
3.5-10, resulted in a similar resolution. Polyacrylamide gels of this separation
are presented in Figure 9. Fraction I is presented on the left for comparison
purposes. Once again, the non-albumin fraction appears in the center, while
the albumin fraction is on the right. Bioassay results indicated that 5 out of 6
animals receiving the albumin fraction hibernated while only 1 out of 6 animals
receiving the non-albumin fraction hibernated. The single animal that hibernated
in the latter group may have done so because of trace contamination of the fraction
with albumin, albumin and a minor component midway in the gel, perhaps transferrin.

The isotachophoretic resolution of 100 mg of hibernating woodchuck plasma is
presented in Figure 10. Two protein peaks, referred to as Fraction 5 and 7, were
assayed for HIT activity. Four out of 6 animals receiving Fraction 7 hibernated
whereas zero out of 6 animals receiving Fraction 5 hibernated. Both Fractions 5
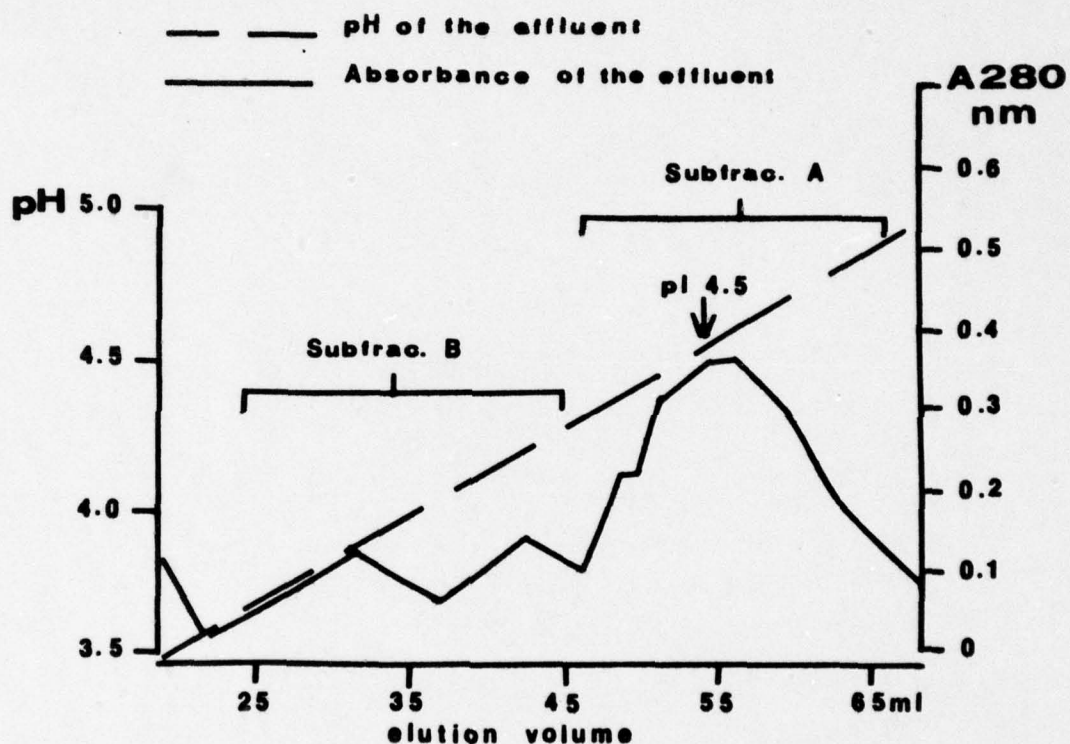and 7 were shown to contain albumin when polyacrylamide gels were run on 400 µg

-12-

Figure 5. Isoelectric focusing at pH 3.5-6.0 of 20 mg of Fraction I obtained by IEF. The pH curve is superimposed.

-13-

Figure 6. Polyacrylamide gels of 400 µg of Subfraction A on the right, Subfraction B in the center, as compared to the starting material, Fraction I, derived by IEF.

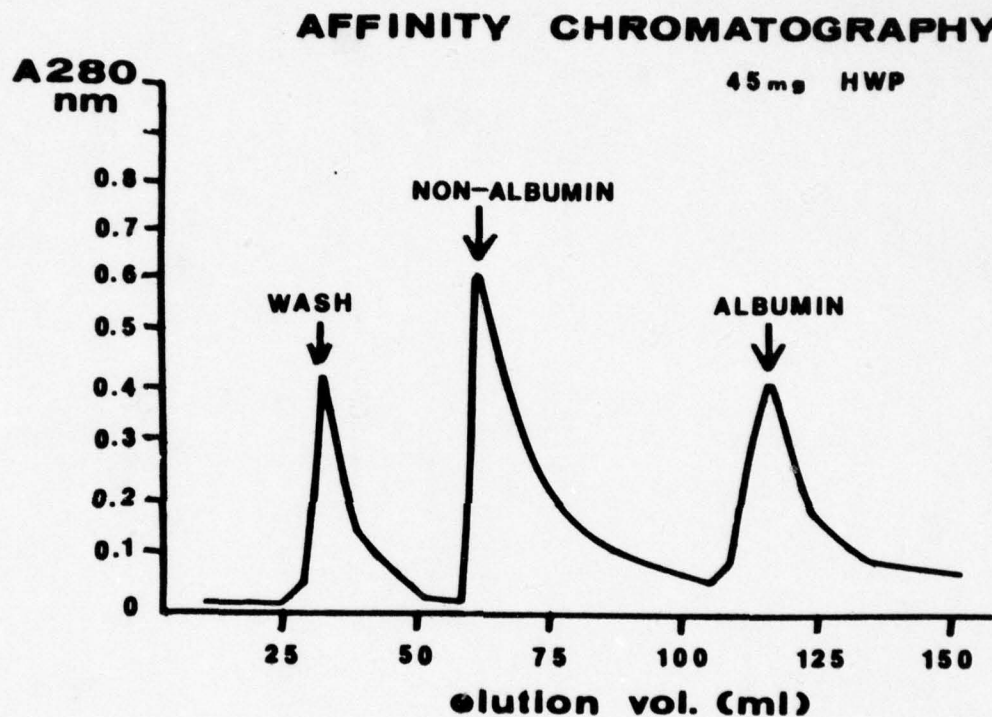-14-

AFFINITY CHROMATOGRAPHY

45 mg HWP

Figure 7. Affinity chromatography of 45 mg of whole hibernating woodchuck plasma resulting in 2 distinct protein fractions. The non-albumin components are right in the center while the albumin component, on the right, is desorbed from the column matrix by a 1.4 N NaCl wash.

-15-

Figure 8. Polyacrylamide gels of 400 µg of an albumin fraction derived from an affinity chromatography column on the right. In the center is the non-albumin fraction from the affinity chromatography column. Both are compared to whole hibernating plasma as seen in the gel on the left.

Figure 9. Polyacrylamide gels of an albumin fraction on the right derived by affinity chromatography. In the center is the non-albumin fraction. The starting material, Fraction I, derived by IEF is shown on the left for comparison purposes.

ISOTACHOPHORESIS
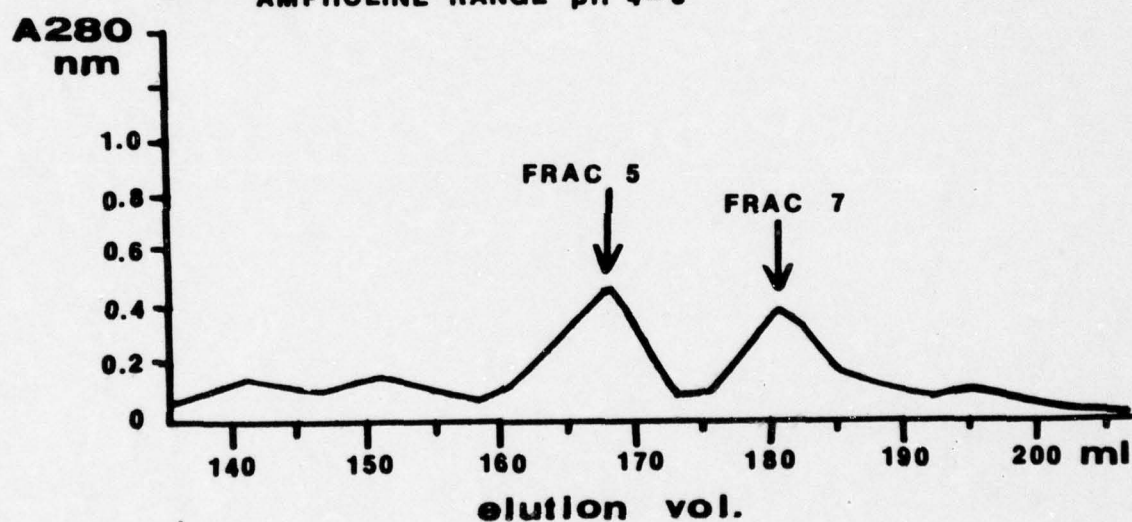
100 mg HWP

AMPHOLINE RANGE pH 4—8

Figure 10. Isotachophoresis of 100 mg of whole hibernating woodchuck plasma. The elution profile at 280 nm indicates several protein peaks. The 2 most prominent ones, Fraction 5 and Fraction 7, were assayed for HIT activity.

-18-

aliquots of these fractions as shown in Figure 11. None of the other anion fractions contained albumin and time, plus a limited number of ground squirrels available for bioassay purposes prevented us from assaying the remainder of these rather non-distinct fractions for HIT activity. Fraction 5 in the center contains a heavy pre-albumin and albumin component plus traces of 4 minor proteins. Fraction 7 on the right contains a trace of pre-albumin, albumin, and dense band midway in the gel of unknown composition. Both of these fractions can be compared to whole hibernating woodchuck plasma on the gel at the left.

Recalling that both Fractions 5 and 7 contain albumin and that HIT activity of affinity chromatographic and IEF separations resided with fractions predominated by albumin, it is possible to theorize that by this technique we may have done one or all of the following things to albumin. One possibility is that we have dissociated the albumin into two components by the specialized resolving charac-teristics of the isotachophoretic procedure which separates ampholytes based on a combination of their electrophoretic mobility characteristics, molecular sieving and pI of those molecules. This could well result in two different types of preparations which were predominated by two slightly different albumin preparations, one an HIT containing component (Fraction 7) and the second (Fraction 5) in which HIT has been dissociated from albumin. A second possibility is that a portion of the albumin preparation and its associated HIT component has been inactivated by the conditions at which Fraction 5 is isolated, i.e., its pH environment has been changed sufficiently by the ampholine chemicals to alter the protein structure of both albumin and its associated HIT. Another less likely possibility is that the HIT activity resides with the bands 4.7 and 4.8 cm above the tracking dye on Fraction 7 (Figure 11). These minor bands may correspond to a single transferrin band of whole hibernating woodchuck plasma as indicated by the polyacrylamide gel on the right in Figure 11. These minor bands are present as trace contaminants with most of the HIT active plasma fractions.

Utilizing three distinct resolving techniques of isoelectric focusing (IEF), isotachophoresis and affinity chromatography has clearly indicated that the probable plasma locus for the HIT molecule resides with the albumin fraction of hibernating woodchucks plasma (Oeltgen et al, 47). The few animals which hibernated when injected with the non-albumin Fraction did show residual amounts of albumin which may have had sufficient associated HIT to induce hibernation even at very low levels. Figure 12 shows all resolved plasma fractions having HIT activity derived from these three techniques as compared to whole hibernating woodchuck plasma which is indicated on the left. The affinity chromatography technique has provided the most nearly homogeneous albumin fractions to exhibit HIT activity. These preparations were comprised almost entirely of albumin and a small amount of pre-albumin and in a few instances traces of transferrin mid way in polyacrylamide gels of these fractions. The resolution of the albumin pre-paration has been rapid and efficient utilizing this technique. Thus, it has been used to obtain starting material for most of the experiments on which we will now report and will be utilized in much of our future experimentation to obtain starting material for additional isolation studies.

b. Isolation Studies & Characterization Studies 1978

Preparative Electrophoresis - Preparative electrophoresis of whole hiber-nating woodchucks plasma or the HIT-active albumin fraction derived from the affinity chromatography column was performed utilizing the LKB 7900 Uniphor
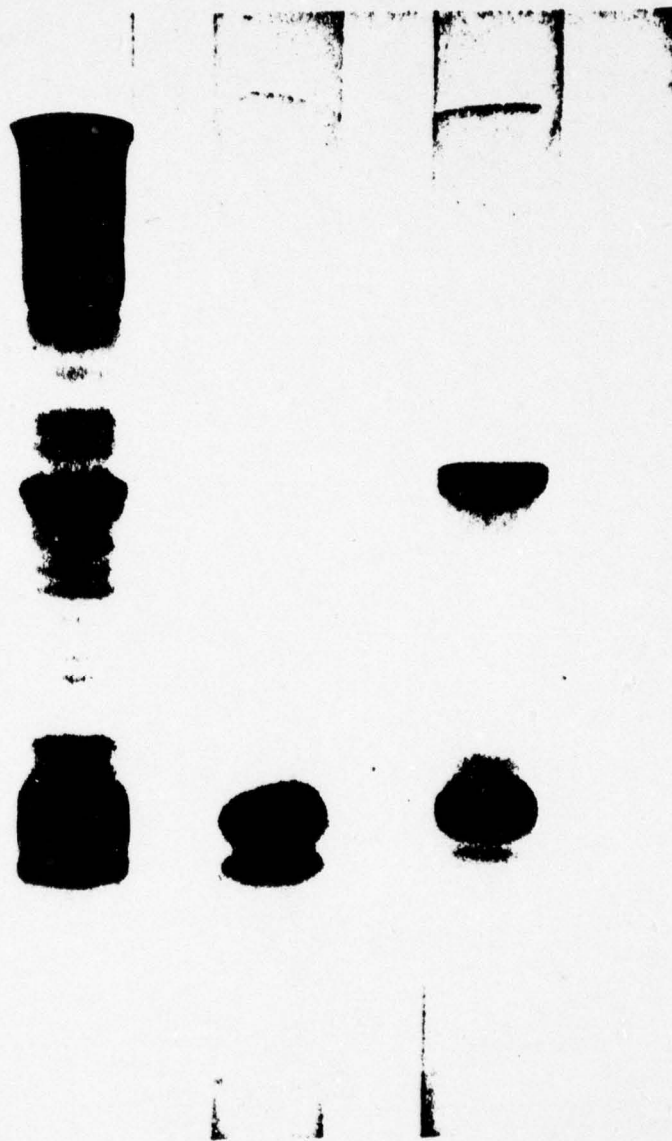
Figure 11. Polyacrylamide gels of 400 µg aliquots of Fraction 7 on the right and Fraction 5 in center derived by isotachophoresis. They are compared to the starting material, whole hibernating woodchuck plasma, as shown on the gel at the left.
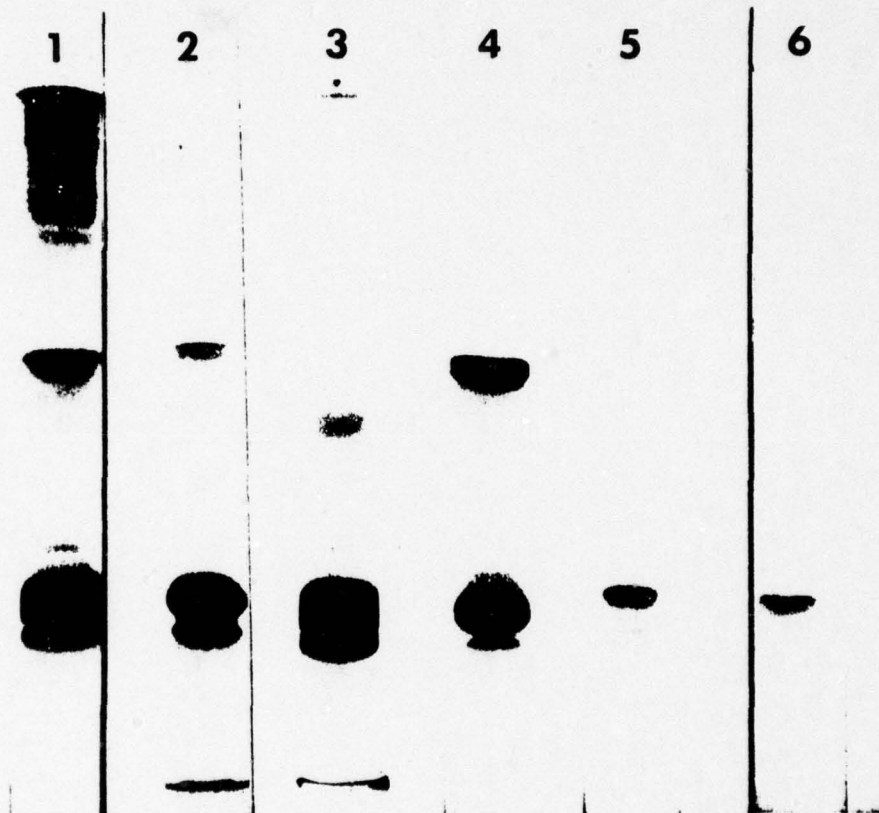
Figure 12. Polyacrylamide gels of 400 µg aliquots obtained from all hibernating woodchuck plasma fractions exhibiting HIT activity obtained in our 1977 isolation studies. These fractions in which albumin predominates were derived by IEF, isotachophoresis and affinity chromatography. Gel number 1 represents whole hibernating woodchuck plasma for comparison purposes. Gel 2 and 3 were derived by IEF and represent Fraction 1 and subfraction A, respectively; Gel 4 represents fraction 7 derived from isotachophoresis column while gels 5 and 6 represent affinity chromatography separations of albumin fractions from whole hibernating woodchuck plasma and Fraction 1, respectively.

-21-

Column (LKB Instruments, Stockholm, Sweden). A 2.5 x 7 cm polyacrylamide gel was formed in the column. Gel solutions were identical to those routinely used for analytical polyacrylamide gel electrophoresis. The buffer was tris-glycine pH 8.3. The sample applied to the gel was 144 mg of combined albumin fractions from the affinity chromatography column mixed in 40% sucrose. The electrophoretic resolution was performed over night at 18 mA with an elution rate of 10 ml per hour. The absorption of the effluent was monitored at 280 nm. Three distinct protein fractions were eluted as shown in Figure 13. We shall refer to these peaks as Fraction 1, 2, 3. Each of these fractions was dialyzed and lyophilized. 400 µg aliquots of each fraction, were inspected for homogeneity by analytical PAGE as shown in Figure 14. Bioassay results indicated that 3 out of 5 summer active ground squirrels hibernated when injected with 3 mg/ml of Fraction 1, while 2 out of 5 hibernated when injected with Fraction 2 and zero out of 5 hibernated when injected with Fraction 3.

A quite similar electrophoretic separation was noted when 100 mg of whole hibernating woodchuck plasma was applied to the preparative PAGE column and resolved under identical conditions. Analytical PAGE of aliquots of this fraction were quite similar to those seen in Figure 4. However, bioassay results were little more impressive. Four out of five animals receiving Fraction 1 hibernated, while 3 out of 5 animals injected with Fraction 2 hibernated and zero out of 5 hibernated when injected with Fraction 3.

It is readily apparent from Figure 14 that Fraction 1 has a single prominent protein band which migrated to a comparable position with the major albumin band in Fraction 2. Fraction 1 has no evidence of either prealbumin on transferrin contamination but it does exhibit a very minor and diffuse band of unknown composition which has migrated beyond the transferrin band seen in Fraction 3. Fraction 2 reveals prominent prealbumin and albumin bands and several minor bands of unknown composition just above albumin. It too, has no evidence of transferrin. As seen in Figure 14 Fraction 3 has a prominent transferrin band midway in the gel and has no evidence of albumin or prealbumin. Since Fraction 3 from both whole hiber-nating woodchuck plasma and affinity chromatography albumin fraction failed to induce hibernation in either of the five animal test groups receiving this fraction, we can effectively rule out transferrin as a possible plasma binding protein for the HIT-molecule. (Oeltgen et.al.(48).

It seems quite possible that the preparative PAGE of the affinity chromatography albumin preparation might have generated a Fraction 1 which represents one of the following: (a) a homogeneous albumin preparation with its HIT molecule still intimately bound or associated or (b) a dissociated and nearly homogeneous HIT molecule which has a similar electrophoretic mobility to that of albumin seen in gels of Fraction 1, Figure 14. The molecular identity of Fraction 1 will be established during the next year by a number of resolution techniques. These include preparative and analytical sodium dodecyl sulphate (SDS) - PAGE, preparative PAGE utilizing 6 M urea in the gels and chromatographic columns utilizing SDS and hydroxylapatite as the chromatography matrix.

c. Dose-Response Study. In the past, limitations in either the quantity of isolated HIT-active plasma fractions or the numbers of available ground squirrels for summer bioassay purposes, had prevented us from conducting a dose-response study. We had no means of knowing whether or not our use of a 3 mg/ml injection dose of resolved plasma fractions from hibernating woodchucks was appropriate. Therefore, summer-active ground squirrels were injected with varying concentrations of our albumin fraction derived from an affinity chromatography column utilizing Affi-Gel Blue. Three dose levels (3 mg/ml, 1.5 mg/ml and 0.75 mg/ml of physiologi-cal saline) were injected into the saphenous vein of 5 animal test groups. Our

PREPARATIVE PAGE
of
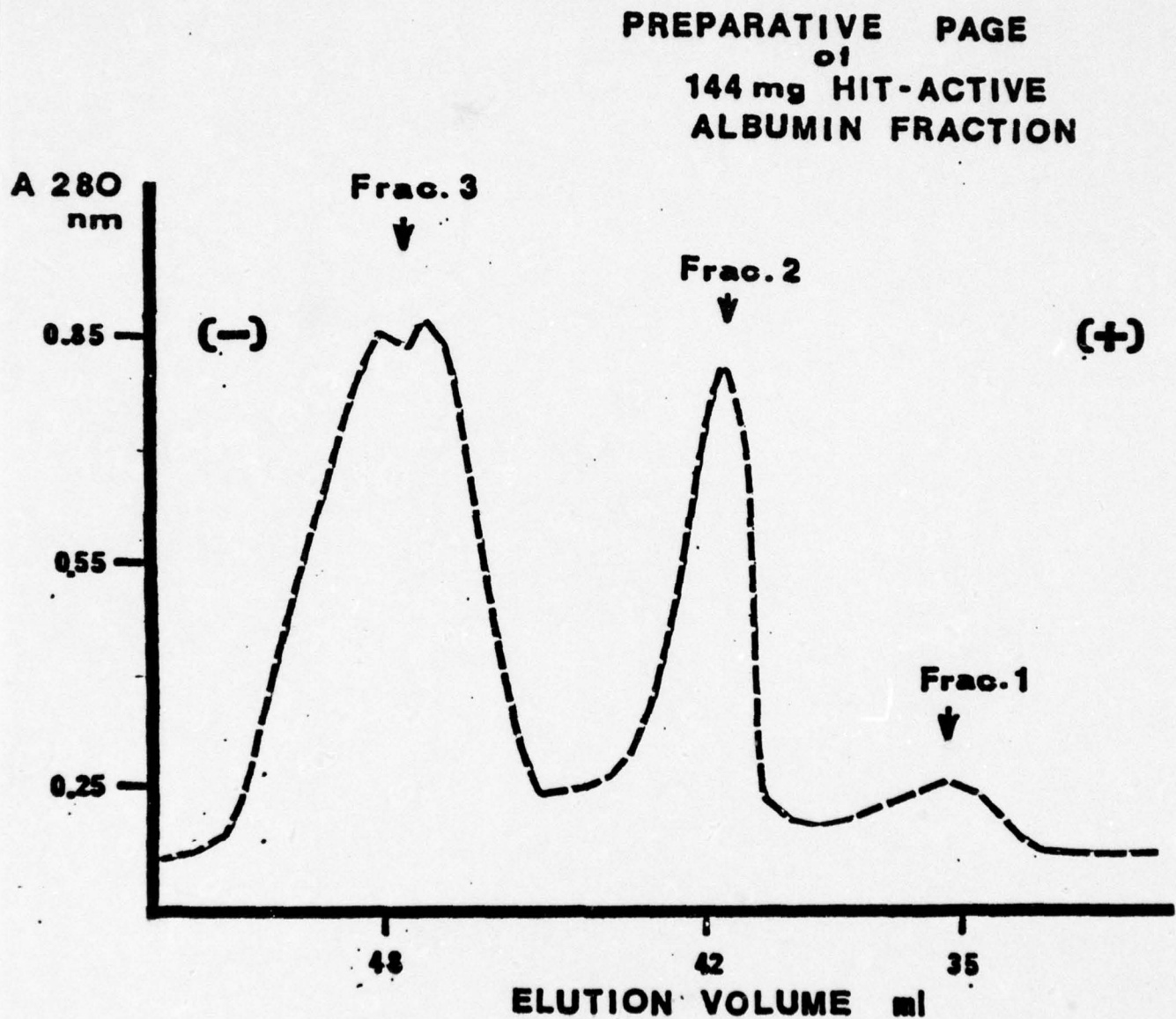144 mg HIT-ACTIVE
ALBUMIN FRACTION

Figure 13. Preparative electrophoretic resolution of 144 ug of HIT-active albumin fraction in an LKB 7900 Uniphor column. The three protein peaks are referred to as Fraction 1, 2, and 3.

Figure 14. Analytical PAGE of 400 μg aliquots of Fractions 1, 2, and 3 which were derived by preparative PAGE on LKB Uniphor as compared to whole hibernating woodchuck plasma.

bioassay results indicated that 5 out of 5 animals receiving the 3 mg/ml dose level hibernated, while 4 out of 5 animals which had received the 1.5 mg/ml dose hibernated, and only 2 out of 5 recipients of the 0.75 mg/ml dose hibernated. This study clearly indicated that our arbitrary choice of a 3 mg/ml injection dose of HIT-active plasma fractions at the beginning of our isolation studies had been fortuitous. Consequently, this dose will continue to be utilized should it be necessary to perform bioassay in summer-active ground squirrels in the future.

d. Inactivation Studies

Heat Inactivation - Much concern has been voiced by other investigators in this field over the thermolabile nature of the blood borne "trigger" which induces natural mammalian hibernation. The early studies of Dawe and Spurrier (38) prompted much of this concern. Their studies utilizing whole hibernating woodchucks plasma indicated that the "trigger" could be inactivated partially at 20°C and completely at 37°C for 30 minutes in vitro prior to injection. Yet, whole hibernating woodchuck plasma maintained at 4°C was quite effective in induction of hibernation in summer-active ground squirrels whose core temperature approximates 37°C. This paradoxical finding is supported by our most recent series of experiments utilizing HIT-active albumin fractions.

In our heat inactivation study, 20 mg of HIT-active albumin fraction derived from an affinity chromatography column was incubated in 5.5 ml of sterile 0.9% saline at 37°C for 30 minutes. Aliquots (3 mg/ml) of this heat-treated fraction were injected immediately therafter into summer-active ground squirrels. Bioassay results indicated that only one out of five animals receiving this heat-treated fraction which had induced hibernation in 5 out of 5 ground squirrels in the dose response study. Therefore, we conclude that inactivation could not be attributed in this instance to anything other than heat. This observation is quite significant and reaffirms our conviction that the HIT molecule is quite thermolabile outside of the test animal. Moreover, an interesting unpublished observation by one of our collaborators, Dr. Jane Roberts at Creighton University, Omaha, Nebraska tends to confirm our findings. In her study, she noticed that much or all of the mitochondrial succinoxidase inhibition by aliquots of hibernating woodchucks plasma disappeared when these preparations were not kept on ice prior to assay or were assayed at 37°C rather than 25°C, the normal assay temperature.

At the present time, we can only speculate on the mechanism of action of the thermolabile HIT molecule in recipient summer-active ground squirrels or woodchucks. Several interesting possibilities come to mind, the first being that the injected HIT molecule may be rapidly sequestered by circulating plasma proteins, specifically albumin. This newly formed macromolecule aggregate may then be protecting the HIT molecule from heat-inactivation and enzymatic destruction by circulating proteases. The long term affect, hibernation induction, may occur gradually as the HIT molecule is released from albumin or other larger protein aggregates and bound to specific receptor organs of tissues. The second possibility is that the hibernator is unique in all the animal kingdom in possessing specialized tissue receptor sites or molecules which immediately bind the HIT molecule and prevent its destruction. Once the HIT molecule is bound to membranes of responsive tissues and organs it may initiate its metabolic inhibitory effect by gradually slowing down one or a series of aerobic mitochondrial enzymic reactions. However, our latest study utilizing monkeys as test animals for the resolved HIT-active fractions tends to rule out this possibility.

(b) Protease and Nuclease Inactivation

If the HIT molecule is truly protein in nature as the heat-inactivation study has lead us to believe, it should be effectively destroyed by pre-incubation with proteases and unaffected by pre-incubation with nucleases. In order to test this hypothesis, 20 mg of the albumin fraction, utilized in the previously cited dose study, was added to 4.0 ml of 0.01 M potassium phosphate buffer at pH 7.0 at 4^0 C. The stock proteases utilized in this experiment was, subtilisin BPN (Sigma Biochemicals, St. Louis, Missouri) at a concentration of 1 mg/ml of buffer. The sample to enzyme ratio was 100:1 and was incubated at 4^0 C for 5 hr. Protease activity was stopped by the addition of p-chloromecuribenzoate. The HIT sample was then dialyzed, lyophilized and injected for bioassay in summer-active ground squirrels. None of the 5 recipients of this protease-treated HIT fraction hiber- nated. Similarly, 20 mg of the same albumin fraction utilized in the dose study were dissolved in 1 ml of 0.1 M $CaCl_2$, 0.6 ml of borate buffer and 0.2 ml of micrococcal nuclease at a concentration of 0.002 mg/ml. This mixture was incu- bated overnight at 4^0 C. Nuclease activity was stopped by the addition of 7% perchloric acid and kept on ice for ten minutes. Five ml of deionized water was added and the sample was immediately dialyzed and then lyophilized prior to injection. Bioassay results indicated that 4 out of 5 animals receiving the nuclease pre-treated HIT fraction hibernated. This effectively ruled out the nucleic acids as possible candidates for the HIT molecule.

Based on the results of the aforementioned heat and enzyme inactivation studies, we have firmly established the thermolability and protein nature of the HIT molecule (Oeltgen et al. 48).

(c) Development of an Assay for Hibernation Induction Trigger(s)

At the present time, the only fully proven assay for the detection of HIT activity of resolved plasma fractions is that of induction of hibernation in summer- active ground squirrels or woodchucks. This places our research involving the chemical characterization of the HIT molecule in a very restrictive time frame. Consequently, a number of attempts are now underway to develop a specific in vivo or in vitro assay for HIT which can be conducted more rapidly and during any time of the year and preferably does not rely on induction of hibernation in summer- active animals.

(1) Hemoglobin Study Alterations

Hemoglobins from fetal (Figure 15) summer-active (Figure 16), summer-hibernating (Figure 17), winter-active (Figure 18) and winter-hibernating (Figure 19) ground squirrels were subjected to isoelectric focusing in order to determine whether or not significant alterations in this molecule occurred with the changing activity states and whether or not there was a reversion to a fetal-type of hemoglobin in either winter or summer-hibernators. A brief summary of our find- ings is given below.

a. The hemoglobin molecule varies markedly with the various activity states of the hibernator.

b. Hemoglobin from summer-hibernating ground squirrels (Figure 17) and winter- hibernating ground squirrels (Figure 19) does not revert to a fetal (Figure 15) type of hemoglobin.
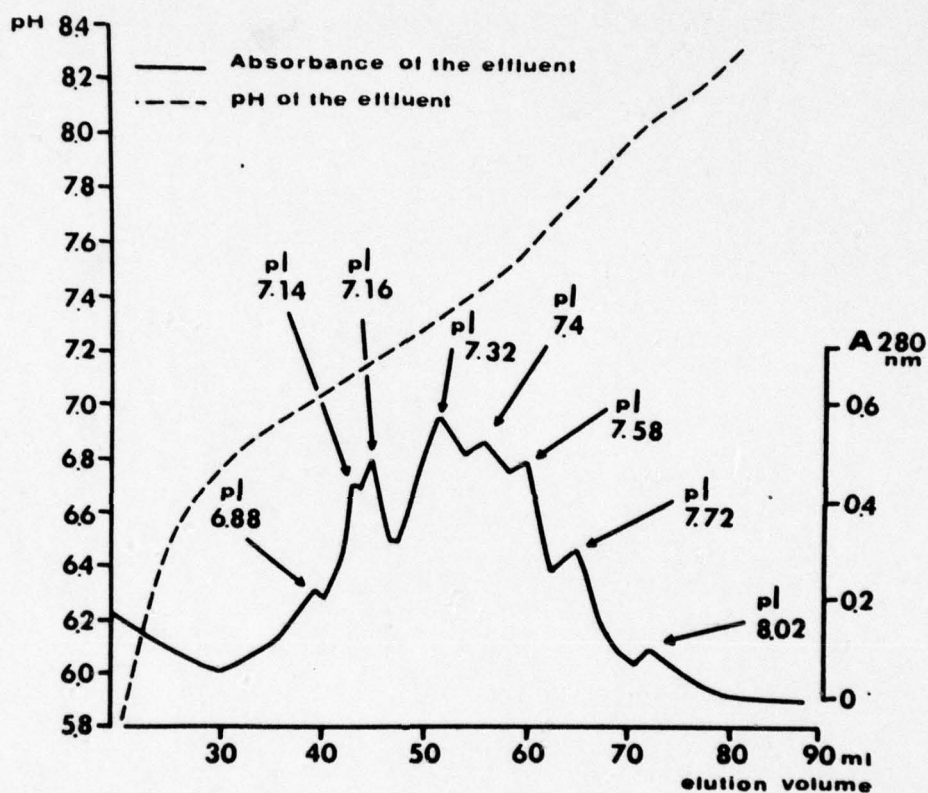
Figure 15. Separation of 20 mg of fetal ground squirrel hemoglobin in a pH gradient extending from 7.0 to 9.0. The curve with peaks shows the absorption in the eluate at 280 nm. The steadily increasing curve is a plot of the pH gradient superimposed. Isoelectric point values shown at the various peaks were read from the pH gradient curve.
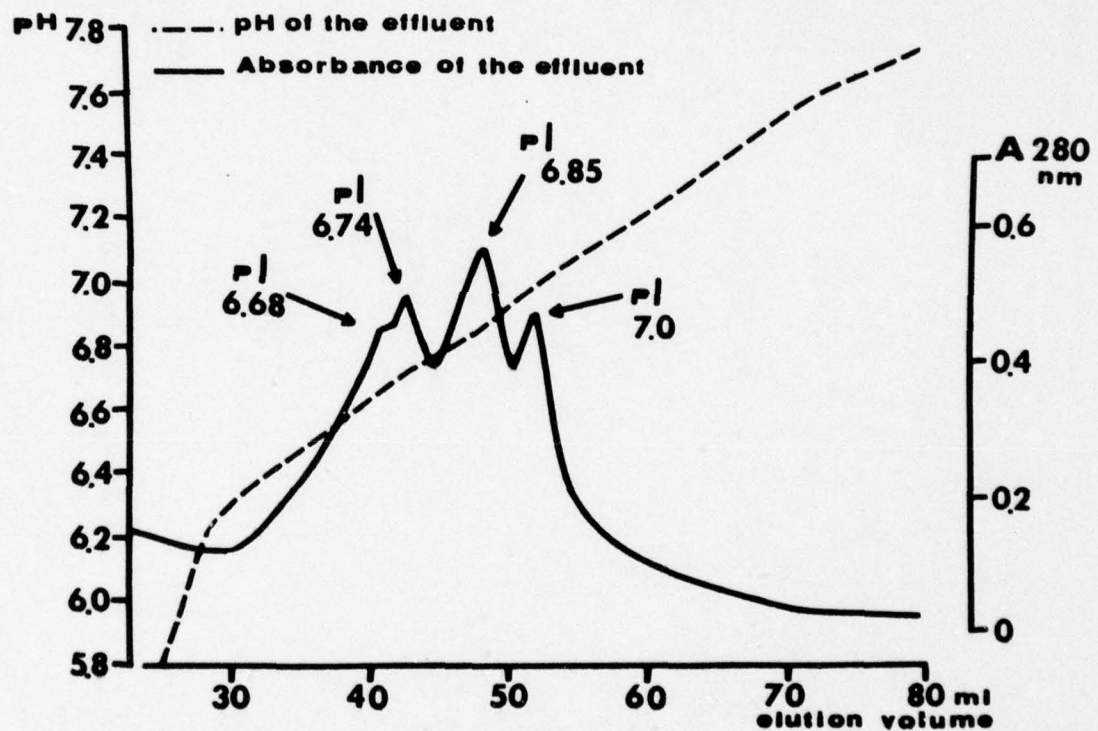
Figure 16. Summer-Active Hemoglobin. Separation of 20 mg of summer-active ground squirrel hemoglobin by the LKB 8100 Electrofocusing Column in a pH gradient extending from 7.0 to 9.0.

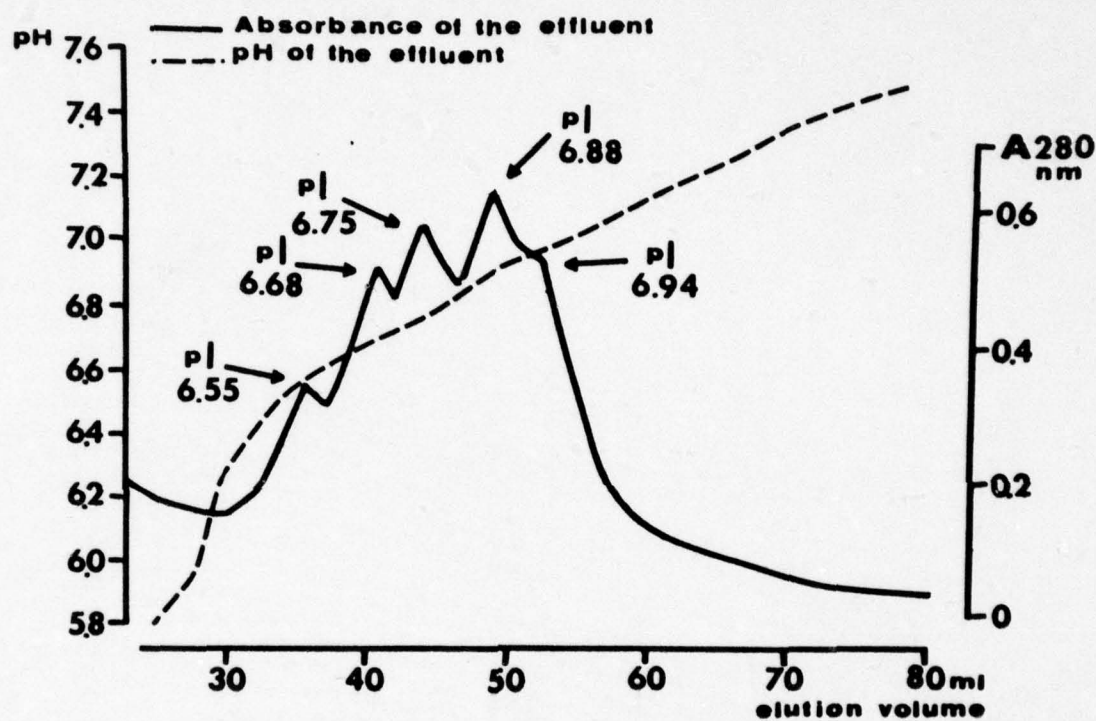-28-

Figure 17. Summer-Hibernating Hemoglobin. Separation of 20 mg of summer-hibernating ground squirrel hemoglobin by the LKB 8100 Electrofocusing Column in a pH gradient extending from 7.0 to 9.0.
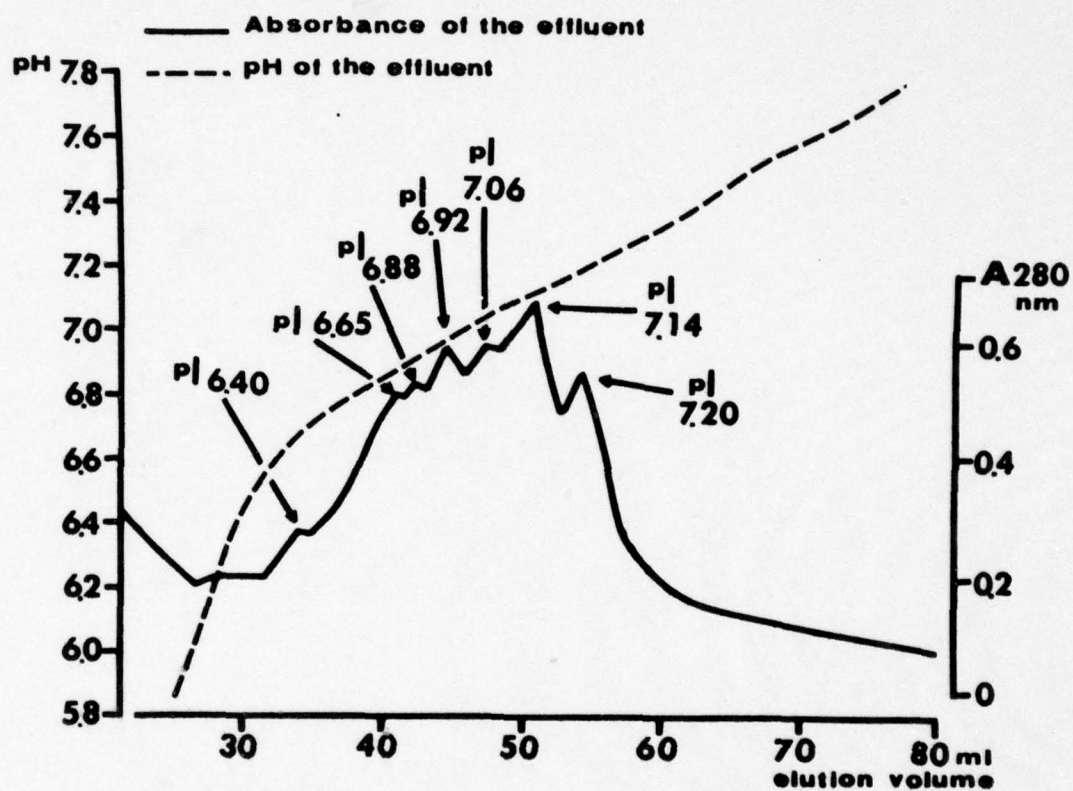
Figure 18. <u>Winter-Active Hemoglobin.</u> Separation of 20 mg of winter-active ground squirrel hemoglobin by the LKB 8100 Electrofocusing Column in a pH gradient extending from 7.0 to 9.0.
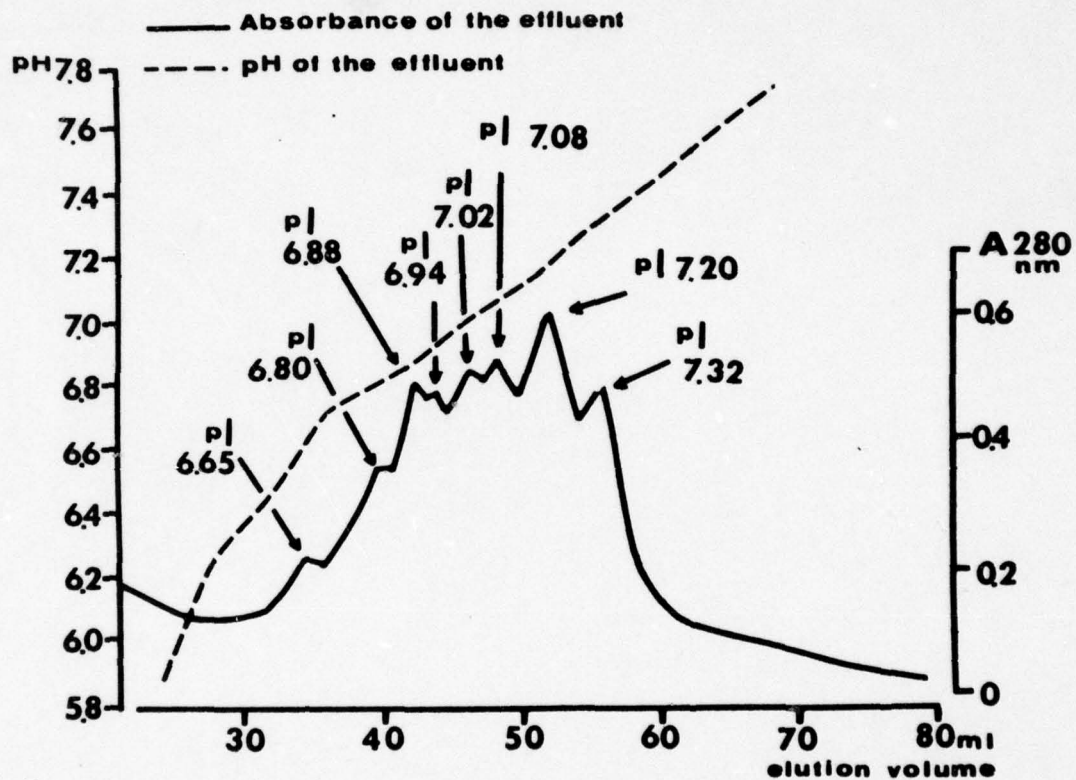
-30-

Figure 19. Winter-Hibernating Hemoglobin. Separation of 20 mg of winter-hibernating ground squirrel hemoglobin by the LKB 8100 Electrofocusing Column in a pH gradient extending from 7.0 to 9.0.

c. A major change is noted in number of hemoglobin peaks as these hibernators go from summer to winter season regardless of the activity state (hibernating or active).

d. Hemoglobin from summer-active (Figure 16) and summer-hibernating (Figure 17) ground squirrels have 4 and 5 hemoglobin peaks, respectively, and their elution profiles are virtually superimposible.

e. Hemoglobin from winter-active (Figure 18) and winter-hibernating (Figure 19) ground squirrels have 7 and 8 hemoglobin peaks, respectively, and their elution profiles are virtually superimposible.

f. An additional hemoglobin peak is seen in summer-hibernating (Figure 17) ground squirrels at pI 6.55 and winter-hibernating (Figure 19) ground squirrels at pI 6.80 which is not seen in the summer and winter-active hemoglobins.

The possibility now exists that these additional peaks of winter and summer-hibernating ground squirrel hemoglobins may be directly attributable to the influence of trigger(s) on the erythropoietic tissue of these hibernators and thus might constitute andassay for the HIT molecule (Oeltgen et al. 30).

(2) Development of an In Vitro Mitochondrial Assay for HIT Activity

A second collaborative study is currently underway with Dr. Jane Roberts, Department of Biology, Creighton University. In this study, HIT-active whole or resolved woodchuck plasma samples were added to ground squirrel mitochondrial preparations whose succinoxidase activity was monitored via a polargraph. The succionxidase assay is that of Estabrook (39) utilizing *100 μmoles of succinate* as substrate.

This study has just begun but initial results are quite encouraging. In one instance, the HIT-active woodchuck plasma fraction added to the mitochondrial preparation decreased succinoxidase activity by over 50% while summer-active plasma and bovine serum albumin preparations were without effect.

This promising study may lead to the development of the much needed in vitro assay for HIT-activity.

(3) Assay for HIT Activity in Monkeys

A most promising assay for detecting HIT activity of resolved hibernating woodchucks plasma fractions in primates was initiated between our laboratories at the University of Kentucky, Lexington, Kentucky and University of North Carolina at Chapel Hill, N.C. last year.

This study has clearly indicated that the rodential HIT molecule can induce a number of profound physiological changes when infused into the brain ventricular fluid of recipient monkeys. Moreover, these changes are reproducible and may thus serve as a bioassay for HIT activity of resolved plasma fractions. Should this be the case, it would free us from the seasonally dependent bioassay currently in use.

Three test animals in this truly exciting preliminary study were 6-8 kg female macaque and rhesus monkeys which had three guide cannules implanted above the 3rd brain ventricle. They also had cardiac electrodes implanted for recording heart rate and ECG and a thermister probe for continuous monitoring of core temperature. In addition, feed intake and activity were continuously monitored. The surgical preparation, monitoring, and maintenance of these primates has been thoroughly documented by Myers et al (50). All test substances infused into the ventricular fluid were first dissolved in an artificial cerebrospinal fluid (CSF) developed by Myers (51). The test materials were albumin preparations derived from the affinity chromatography column of either summer-active or winter-hibernating woodchuck plasma. In each case, these preparations had been assayed for HIT activity in hibernators. A bovine serum albumin preparation was also infused into the ventricular fluid of each monkey to determine if any non-specific protein responses could be induced. Each test animal was infused at appropriate time intervals with each of these three protein preparations.

A brief summary of the results of this pioneering study involving our two laboratories is presented below.

(a) The heart rate decreased by 40-50% of baseline values within hours of infusion of the HIT-active albumin preparations. The response was cyclical in nature and lasted for several days to a week.

(b) Core temperature declined from 2 - 3°C and in one animal dropped 8°C. This monkey was rewarmed with heating pads to prevent it from becoming hypothermic.

(c) A definite and most reproducible metabolic effect was observed in all monkeys after infusion of HIT. Feeding ceased in all animals when heart rates and core temperature were declining and resumed when they returned to baseline levels.

(d) The monkey's eyes were closed and their heads drooped when heart rate and core temperature declined giving the appearance of an anesthetized state.

Infusion of Summer-Active Albumin and Bovine Serum Albumin Preparations (3 mg/300 μl CSF).

None of the aforementioned responses observed with infusion of the HIT active albumin preparation were noted. (Myers et al. 52).

4. RATIONALE: A large number of studies have been conducted which have described the physiological function and regulation of systems relating to a variety of tissues and organs of hibernators (4, 5, 9, 11, 12-14, 18-20). However, the area of direct biochemical characterization of the blood-borne trigger first described by Dawe and Spurrier (36) has, until our recent work, (Oeltgen et al. 47, 48), been virtually unexplored. Perhaps, the primary reason for this has been the requirement of utilizing a bioassay requiring induction of hibernation in summer-active ground squirrels or woodchucks (a very restrictive seasonal time frame for testing)

and the necessity for maintaining these animals in a special hibernaculum where they are exposed to minimal disturbances.

In spite of these major drawbacks, our work over the past 3 years, utilizing three distinct protein resolving techniques of isoelectric focusing (42), preparative isotachophoresis (45,46) and affinity chromatography (43,44) has given the first real clues to the chemical identity of the HIT molecule. Our experiments have demonstrated that this molecule is closely associated with albumin and that its physiological role in hibernators may be dependent upon changing albumin concentrations (23). To date, our studies indicate that the HIT molecule is a small, thermolabile, protease-sensitive protein in excess of 5,000 M.W. However, we have not yet been able to isolate what we consider to be a completely homogeneous preparation which is suitable for molecular weight estimations, amino acid analysis and sequence studies. In addition to these important biochemical findings, our attempts at developing a more convenient bioassay for detecting HIT activity of resolved plasma fractions have given the first indications that hibernators are not unique in their ability to respond to the introduction of this molecule. Preliminary studies utilizing primates may have provided direction to a most suitable bioassay system for testing HIT activity of resolved plasma fractions. Moreover, these studies indicate possible far-ranging clinical applications of this molecule in non-hibernators.

Thus, in order to more fully understand the physiological and biochemical factors regulating natural mammalian hibernation and the metabolic depressant effects in non-hibernators, it is essential that the HIT molecule be completely chemically characterized and the organ or tissue involved in its production be identified.

With the foregoing observations in mind, we intend to complete the biochemical characterization of the HIT molecule and identify the site of its production in hibernators. The first step in the process will be to completely desalt all of our plasma preparations utilizing a hollow fiber or ultrafiltration device. This will be followed by resolving the desalted plasma fractions utilizing the highly efficient affinity chromatography technique to generate HIT-active albumin preparations. These HIT active fractions are comprised primarily of albumin, small amounts of pre-albumin and a trace of transferrin. Our next isolation objective will be to remove the contaminating transferrin and pre-albumin proteins. We feel this can be accomplished by further fractionation utilizing the preparative gel electrophoresis technique. In fact, in one preliminary experiment, we have demonstrated by analytical polyacrylamide gel electrophoresis that the preparative gel technique may generate a homogeneous HIT-active preparation. In order to determine whether or not this single protein peak is indeed the homogeneous HIT molecule, or instead, an albumin molecule which still has the HIT molecule bound to it, we will subject this protein fraction to several non-destructive dissociation techniques. These include preparative sodium dodecyl sulphate (SDS) electrophoresis, preparative gel electrophoresis with gels containing 6 M urea and SDS-hydroxylapatite chromatographic separations. Should our HIT-active preparation remain homogeneous after being subjected to the aforementioned dissociation techniques, we will then utilize analytical SDS-polyacrylamide gel electrophoresis and analytical ultracentrifugation to estimate its molecular weight. In addition, analytical isotachophoresis technique will be emplored to accurately determine a physical constant of the HIT molecule, its isoelectric point

Short term goals also require that we determine the site of production of the HIT molecule in hibernating animals. This can be accomplished by injecting aliquots of resolved HIT-active preparations into rabbits for anitbody production. At

appropriate time intervals, antibody to the HIT molecule will be harvested, purified and labeled with a fluorescent dye. The labeled antibody preparation will then be overlaid on selected tissue slices from hibernating woodchucks or ground squirrels and examined microscopically for immunofluorescent sites indicating HIT production.

Our long-term biochemical characterization will be to determine the amino acid composition and sequence of the pure HIT molecule and possibly chemical synthesis of the molecule. We also intend to perfect our bioassay for HIT activity utilizing primates and to fully explore the clinical potential of this molecule when introduced into non-hibernators.

B. SPECIFIC AIMS:

The specific aims of this proposal are as follows:

Short Term

1. To isolate a homogeneous hibernation induction trigger (HIT) molecule from the plasma of hibernating woodchucks and ground squirrels.

2. To develop a rapid and specific in vitro and/or in vivo assay for detecting HIT-activity of resolved plasma fractions, preferably utilizing non-hibernators.

3. To immunochemically determine the site of production of the HIT molecule in woodchucks and ground squirrels.

Long Term

4. To completely chemically characterize the isolated HIT molecule. This includes determining such physical constants as its isoelectric point and molecular weight. Ultimately, we intend to determine the amino acid composition and sequence of the molecule. Should these objectives be achieved, the last step in the process would be to chemically synthesize the HIT molecule and determine if the synthesized protein exhibited HIT activity in either summer-active hibernators or preferentially in non-hibernating recipients such as primates.

5. Since preliminary observations with prima es which have had HIT preparations infused into their brains have given the first indication that the HIT molecule (now perhaps more appropriately referred to as hypothermia inducing trigger) may initiate transient yet profound biochemical and physiological alterations in non-hibernating recipients. Thus, the full clinical potential of the HIT molecule in such diverse areas as organ preservation and transplantation, cryobiology, anesthesiology and tumor inhibition will be fully explored in both our laboratories and through collaborative research efforts with other investigators.

C. METHODS OF PROCEDURE

To date biochemical characterization of the HIT molecule indicates the following:

1) The HIT molecule is a small, thermolabile protease inactivated protein in excess of 5,000 MW which is transported in the plasma of hibernators either bound to or closely associated with albumin.

2) Evidence from preparative polyacrylamide gel electrophoresis and preparative isotachophoresis experiments tends to indicate that the molecular sieving effect of these preparative gels may dissociate a significant portion of the HIT molecule from albumin.

With this in mind, our most immediate research goals will continue to be focused on the dissociation of the HIT molecule from albumin and isolation of a homogeneous HIT preparation. We will also utilize immunological procedures to monitor the homogeneity of our isolated HIT-plasma fractions and to determine the site of production of this molecule in hibernators. Another major emphasis of our research will be placed on the development of a routine assay for resolved HIT preparations which does not require induction of hibernation in summer-active ground squirrels on woodchucks. Long-term research efforts will be directed at the complete chemical characterization (amino acid composition and sequence) of a homogeneous HIT molecule and determining the full clinical significance of this molecule when infused into the brain ventricular fluid of non-hibernators.

1. Sample Procurement. Plasma to be assayed for HIT activity is obtained from woodchucks and ground squirrels which are maintained in a hibernaculum at $5^{o}C$ in the laboratory of Dr. Edgar Folk, Jr., in the Department of Physiology and Biophysics at the University of Iowa. Until the past year blood from hibernating animals had always been provided for isolation studies by W.A. Spurrier then a research associate in the Department of Physiology, Loyala University, Stritch School of Medicine. However, with her leaving that department the large hibernaculum housing 20 woodchucks and over 400 ground squirrels was most unfortunately shut down. This has spurred our search to develop an assay for HIT activity of resolved plasma fractions which does not require summer active hibernators for bioassay.

2. Bioassay for HIT Utilizing Hibernators. Induction of summer hibernation has been until recently, the assay method utilized for testing whole plasma and fractionated plasma samples for HIT activity. A group of non-transfused animals is always maintained under similar conditions as the experimentals; these animals serve as controls. One year old, summer-active ground squirrels, weighing 260-270 grams and maintained at $5^{o}C$ in the hibernaculum are the test animals for resolved HIT plasma fractions. Plasma sample to be assayed for trigger are injected into the saphenous vein of ground squirrels at a concentration of 3 mg/ml in 0.9% saline, Induction of summer hibernation by the HIT molecule may occur as soon as 2 days to as long as 2 weeks after injection of the HIT preparation.

3. Bioassay for HIT Utilizing Primates. By directly perfusing the cerebral ventricles of primates with resolved plasma fractions from hibernating woodchucks, we can circumvent the blood brain barrier and thus readily determine if a resolved plasma fraction exhibits HIT activity. The presence or absence of HIT activity can be determined by on line monitoring of a number of physiological responses of the animal which is followed concomitantly. Preliminary observations in 3 monkeys indicate reproducible physiological responses (cessation of feeding, hypothermia, and decreased heart rate) occur when their lateral brain ventricles are infused with resolved plasma fractions of hibernating woodchucks shown to have HIT activity in summer-active ground squirrels. None of the aforementioned physiological responses occur when similarly resolved fractions from summer-active woodchucks on bovine serum albumin are infused.

(a) Primate Surgical Procedure - Each of three species of macaque monkey, M. nemestrina, M. iris or M. mulatta, weighing 6-7 Kg is acclimatized to a special restraining chair before an experiment begins, and is trained to pull a lever to obtain food pellets. The surgical and other experimental methods are carried out under sodium pentobarbitone anaesthesia (30 mg/Kg) injected into the saphenous vein with rigid aseptic precautions and following surgical procedure described previously (Myers 53 and Myers et. al. 54). An indwelling array of 4 to 8 guide tubes of a special design is implanted chronically in the brain, utilizing aseptic precautions. Each tube rests just above one region of the hypothalamus, thalamus, mesencephalon or other brainstem structure. Typically, the guide tube is used for more than one type of experiment, i.e., for the microinjection of the HIT molecule or other chemical, the push-pull perfusion of a solution, or for the insertion of a thermode, an electrode or thermistor bead. In addition, a thermistor bead is implanted so as to rest against the sagittal sinus, and in selected cases EEG electrodes for recording cortical potentials are inserted in the calvarium of the monkey. A polyethylene pedestal attached to the calvarium is used to protect the entire array and to maintain a sterile preparation.

In collaboration with the temperature group in the Medical School at Calgary, Canada, we have developed an encapsulated subminiature transmitter which contains a temperature-sensitive diode utilized for telemetering purposes. This implantable package is positioned permanently in the peritoneal space of the monkey. A demodulator system has been developed in our Laboratory so that it is interfaced directly with a multi-channeled potentiometric recorder. This now enables us to record body temperature of the monkey continuously for up to six months (the life of the individual battery). A channel for heart rate has been incorporated in the larger transmitter suitable for the monkey. With the development of this telemetering device, and the usage of on-line pCO_2 and pO_2 instruments, we shall be able to monitor the monkey's temperature and other vital physiological processes without disturbance at any time during the course of an experiment.

(b) Perfusion Procedure - Before a perfusion of resolved plasma fractions commences, the diaphragm of each guide tube cap is cleansed with 70% alcohol. The inner inflow cannula is then lowered through the diaphragm into the guide tube to a point at which flow is established into the lateral ventricle.

A variable-speed Harvard model 935 infusion withdrawal pump is used (Harvard Apparatus, Inc., Millis, Mass.) for perfusion of HIT containing solution. As soon as the fluid flows freely from the ventricle, the pump is started and perfusion is carried out at a rate of 200 ul/min. In our preliminary experiments we have arbitrarily decided upon an infusion dose of 3 mg/300 ul of perfusion of resolved plasma fractions. An artificial cerebrospinal fluid containing Na+, 127.65 mM; K+, 2.55 mM; Ca++, 1.26 mM; Mg++, 0.93mM; and Cl$_2$⁻, 134.58 mM (Myers et al. 55) is used for all perfusions. The position of the ventricular cannulas is verified according to standard methods described by Myers et.al. (54).

4. Desalting and Concentrating of Hibernation Plasma - Desalting of plasma samples will be accomplished using a Bio-Fiber 50 Beaker which has a nominal molecular weight cut off of 5,000. Utilizing this hollow fiber device available from Bio-Rad Laboratories (Richmond, California), low molecular weight solutes such as glucose, urea, catecholamines, and salts are completely removed after 2 hours of passing dialysate at a flow rate of 100 ml/minute. Concentration of hibernation plasma can also be accomplished using either the Bio-Fiber 50 Beaker or the Bio-Fiber 80 Beaker with a molecular weight cut off of 30,000.

5. Protein Determinations - Protein content of resolved plasma fractions will be determined using the method of Lowry et al. (56) as modified by Oyama and Eagle (57).

6. Polyacrylamide Gel Electrophoresis (PAGE) - The homogeneity of resolved plasma fractions derived from such preparative procedures as isoelectric focusing, preparative isotachophoresis or gel electrophoresis, affinity chromatography or hydroxylapatic columns will be monitored by polacrylamide gel electrophoresis using the standard methodology of Davis (58). Electrophoretic separation of 400 µg of both whole and fractionated plasma samples will be performed in 7.5% (w/v) gels. The samples will be run in 5 x 127 mm tubes at a constant current of 2.5 milliamperes pen gel. The resolved gels are stained with Coomassie Blue according to the method of Chrambach et al. (59). The protein patterns of resolved gels are either scanned with a densitometer or photographed.

7. Analytical Isotachophoresis - Analytical isotachophoresis will be carried out on affinity chromatography albumin preparations having HIT activity. We will closely follow the protocol described by Drysdale et al (60). This should give us an accurate indication of the isoelectric point (pI) range of all protein components associated with this fraction and any other resolved plasma fractions thought to have HIT activity. Utilizing this technique will allow us to optimize the buffer pH for future preparative polyacrylamide gel electrophoresis (PAGE) separations of the HIT molecule.

8. Analytical and Preparative Sodium Dodecyl Sulphate (SDS) - PAGE - The HIT active affinity chromatography albumin preparations will be subjected to analytical SDS-PAGE following modifications of the procedures described by Shapiro and Maizel (61), Dunker and Rueckert (62); Swank and Munkres (63) and Gordon (64). Treatment of the affinity chromatography derived albumin fraction with the detergent, SDS, and a reducing agent should be effective in changing the compact 3-dimensional shape of albumin and other associated proteins into rod-like proteins as described by Reynolds and Tanford (65). Righetti and Drysdale (66) have shown that the change

per unit weight of the SDS-treated proteins thus becomes constant and electrophoretic mobility now is solely a function of the molecular weight. Moreover, this SDS-pretreatment may be effective in completely dissociating the HIT molecule from albumin. This may also give us an indication of the range of molecular weights of proteins comprising the HIT-active sample and will permit optimization of gels strengths for preparative SDS-PAGE isolations of the HIT molecule. Molecular weight determinations of homogeneous HIT-active protein fractions derived by preparative SDS-PAGE will be performed utilizing 10% (W/V) SDS and 0.1M phosphate buffer, pH 7.2 as described by Shapiro and Maizel (61) and Swank and Munkries (63).

9. <u>Affinity Chromatography</u>. In this technique, a 15.5 x 1 cm affinity chromatography column was packed with a 10 ml bed volume of Affi-Gel Blue (Bio-Rad Industries, Richmond, California). The Affi-Gel Blue is highly effective affinity chromatography matrix comprised of a reactive blue dye (Cibacron Blue F36A) cross-linked to agarose beads. Affi-Gel Blue has been utilized by Travis and Pannell (43) and Wille (44) to selectively absorb albumin from human serum. In our experimental protocol, carried out at 4°C, the column was initially washed with 0.02 M Na-phosphate buffer pH 6.8. An equivalent of 45 mg was applied to one column. Fifty-five milligrams of lyophilized Fraction I, obtained by IEF, was dissolved in the 0.02 M Na-phosphate buffer pH 6.8 and similarly applied to another column. Both Fraction I and whole hibernating woodchuck plasma were eluted from the column using 0.02 M Na-phosphate buffer pH 6.8 followed by 0.02 M Na-phosphate buffer pH 5.7. The effluent from this step yielded the non-albumin fraction (predominantly gamma globulins). A high concentration of salt, 1.4 M NaCl, added to the pH 5.7 buffer was then utilized to desorb the albumin fraction of the plasma from the gel matrix. The column was then regenerated with 2 bed volumes of 6 M quanidine HCl. The eluted albumin and non-albumin samples will be desalted and lyophilized prior to testing for HIT activity.

10. <u>PAGE Utilizing 6M Urea</u> - Preparative polyacrylamide gels will be cast as previously described (45) in the LKB 7900 Uniphor column. However, 6 M urea will be added to these preparative gels in an attempt to completely dissociate the HIT molecule from albumin. The 6 M urea can be readily removed by Spectrapor dialysis tubing or by utilizing a Bio-Fiber 20 Beaker (Bio-Rad, Industries, Richmond Calif.)

11. <u>SDS - Hydroxylapatite Chromatography</u> - Hydroxylapatite, a form of calcium phosphate, is widely used in preparative enzyme and other macromolecular separation. This technique often resolves complex substances when all other separation methods fail and multiple components are often obtained from proteins shown to be "homogeneous" by other means such as ion exchange chromatography or electrophoresis. Consequently, we will try to completely dissociate HIT from affinity chromatography albumin preparations which have been pretreated with SDS and are applied to columns having hydroxylapitite as the chromatography matrix. Moss and Rosenblum (67) have demonstrated high resolution of viral peptides from complex mixtures utilizing this technique.

12. <u>Immunological Identification and Quantitation of the HIT molecule</u> - Rabbits will be injected at specified time intervals with HIT-active albumin fractions derived from an affinity chromatography column. The antisera harvested from these rabbits will then be utilized for gross immunological identification of the HIT molecule utilizing the double immunodiffusion technique of Ouchterlony (68) If we are successful in dissociating the HIT molecule from albumin, we will monitor the efficiency of our separation and specifically identify and quantitate the HIT antigen by utilizing the rocket immunoelectrophoresis technique developed by Laurell (69) and two dimensional immunoelectrophoresis technique described by Wright <u>et al</u>

(70). These quantitative immunoelectrophoretic techniques will be performed on·the LKB Multiphor utilizing its immunoelectrophoresis kit (LKB Instruments, Stockholm, Sweden). Both of these techniques are based on the electrophoresis of the antigenic HIT molecule into an agarose gel which already contains HIT-specific antibodies formed in rabbits injected with the various HIT-active fractions. Precipitin lines are generated in the agarose gel as the electrophoretically resolved HIT antigen inter-acts with these antibodies specific for it. The height of the rocket shaped preci-pitin line is proportional to the amount of HIT antigen in the applied sample, and the position of the line in the double immunodiffusion technique serves to identify the HIT Molecule.

13. Immunofluorescent Identification of the HIT Molecule in Hibernators. We intend to identify the organ on tissue responsible for synthesis of the HIT molecule in hibernators by utilizing an immunofluorescent technique. The most interesting work of Swan and Schatte (16) who isolated a crude metabolic depressant protein from subcortical brain tissue of hibernating ground squirrels indicates that this tissue should receive first priority in this type of study.

Other organs and tissues which will be examined by this technique are the adrenal gland based on the work of Popovic and Vidovic (11), Kayser and Petrovic (12), the pancreas based on work of Laufenberger (8) Dworking and Finney (9) and Suomalainess (10), and brown fat based on the work of Rassmussen (3) Johansson (4) and Goldman and Bigelow (5).

The immunofluorescent technique first utilized successfully by Coons et al (71, 72) and later refined by Riggs et al. (73), Marshall et. al. (74) and Goldstein et al. (75) combines histochemical and immunological methods to pinpoint specific antigen-antibody complexes present in tissue sections with the aid of a fluorochrome (fluorescein isothiocyanate (FITC).

Briefly, our plan is to innoculate rabbits with an albumin fraction from affi-nity chromatography separation homogeneous preparative polyamylamide gel fraction having HIT activity as indicated by bioassay. After an appropriate interval sera will be collected from these rabbits and the IgG fraction will be purified by ammo-nium sulfate precipitation utilizing a 50% saturated ammonium sulfate or by affinity chromatography utilizing AffiGel Blue as the chromatography matrix to remove albumin. The purity of the fraction will be checked by immunoelectrophoresis. A pure IgG antibody preparation will then be obtained by preparative gel electropho-resis and/or chromatography with DEAE cellulose. The purified IgG antibody to the HIT molecule will then be labeled to FITC by allowing the dye to react with an alkaline solution of HIT antibody protein as described by Marshall et.al. (74). Tissues from hibernating woodchucks which will be examined as possible sites of production for the HIT molecule will rapidly frozen at-80C in a REVCO Freezer after the animal is sacrificed and sectioned by a crystal (available to Oeltgen in the Clinical Laboratory, VA Medical Center). The frozen tissue sections fixed in 100% ethanol are then overlaid with the labeled HIT antibody and the excess is washed off. The tissues are then examined under a fluorescence microscope for fluorescence which indicates the presence of an HIT antigen molecule and most likely the site of production of the HIT molecule.

14. Amino Acid Analysis and Sequencing - Should we be successful in isolating a homogeneous HIT preparation by any of the aforementioned techniques an aliquot of that material will be sent to AAA Laboratory, 6206 89th Ave. Southeast Mercer Island, Washington 98040. This laboratory has an excellent reputation and does all the amino acid analysis and sequencing for several pharmaceutical firms including Armour Pharmaceutical, Kankakee, Ill. We will also perform some amino acid analysis of our own on acid hydrolyzed HIT fragments utilizing the LKB Tachophor.

D. SIGNIFICANCE: The complete chemical characterization of a molecule which can induce or "trigger" natural mammalian hibernation may have far-ranging implications. Characterization of the HIT molecule would provide a more comprehensive understanding of the biochemical basis for this more remarkable physiological phenomenon and an opportunity to explore the clinical potential of this molecule in non-hibernating recipients.

Let us examine some of the ramifications or physiological significance the HIT molecule would have on the circannual events of a hibernator. It is well documented that man and other mammals that are non-hibernators are unable to resist the lethal effects of very low body temperature on cells in organs whereas the hibernator can survive lowering of body temperature to near zero without tissue injury or interruption of vital physiological functions. Evidence is available that the HIT molecule may have a direct action on protein and/or lipoprotein synthesis thereby preparing active animals to survive extended hypothermic episodes. This is demonstrated by our own studies with ground squirrel hemoglobin (Oeltgen et al. 30) which indicate that the erythropoietic tissues of ground squirrels are responsive not only to seasonal changes but also to the direct presence of the HIT molecule.

It is our feeling that in the depressed metabolism noted during hibernation does not represent a reversion to an anaerobic type of metabolism and that the erythropoietic tissues respond by synthesizing hemoglobin molecules which can more readily dissociate oxygen to hypothermic tissues. This may be the role of the additional hemoglobin peaks in both natural and summer-induced hibernating ground squirrels. It is also likely that alterations in erythrocyte membrane protein and lipoprotein are initiated by the presence of the HIT molecule. Spurrier and Dawe (22) have reported a "folded-over" appearance of erythrocytes in hibernating ground squirrel blood and increased resistance of erythrocytes in osmotic fragility tests. These investigators proposed that the "folded over" erythrocytes were more pliable than normal and were thus able to circulate more readily through constricted blood vessels seen in hibernation. Our own preliminary studies of yearly metabolic profiles of individual woodchucks (unpublished) indicate a predominance of the aerobic LDH_1 isoenzyme in the plasma with almost no LDH_4 and LDH_5 in both natural winter and summer-induced hibernating woodchucks. In contrast, winter and summer-active animals have a preponderance of LDH_2 isoenzyme and elevations of LDH_4 and LDH_5. Such evidence indicates that the HIT molecule may initiate the synthesis of isoenzymes which are generally associated in highest quantities with aerobic tissues. Similar types of observations have been reported by Moons (76) and Behrisch (77) and are counter to the observations of Johnsson and Senturia (78) who suggest changes in LDH isoenzymes in myocardial tissue are consistant with a displacement toward an anaerobic metabolism during hibernation. Our yearly metabolic profile also indicates dramatic shifts in lipoprotein composition of woodchucks. The Beta and Prebeta lipoproteins predominate during winter-active and summer-active periods and alpha lipoproteins predominate during winter and summer-induced hibernators. Alterations in protein synthesis especially isoenzymes as well as membrane proteins or lipoproteins may well occur when tissues of hibernators are exposed to the HIT molecule. Thus, synthesis of proteins or lipoproteins which would maintain the flexability of cellular membranes and prevent rapid ion shifts would be of great survival benefit for these animals during extended hypothermic periods.

The clinical potential of a molecule such as HIT which can depress the metabolism of non-hibernators is vast. For example, Goldman and Bigelow (5) have shown that rabbits injected with plasma of hibernating animals exhibited a marked lowering in the critical temperature at which ventricular fibrillation occurred. Chute (79) has demonstrated increased resistance of hibernating host to specific microbial protozoan and metazoan infections. Lyman and Fawcett (80) have shown inhibition of sarcoma development during hibernation and Mussachia and Barr (81) have demonstrated that the resistance of hibernating ground squirrels to normally lethal radiation doses, as is indicated by their higher LD50 and longer mean survival time compared to summer-active ground squirrels.

We feel the areas of organ preservation and transplantation in non-hibernators following brain infusion of the HIT molecule should also be thoroughly explored as well as its role in cryosurgery (Armour et al. 82) and tumor inhibition.

The work of Swan and Schätte (16) utilizing crude protein extracts from subcortical brain tissue of hibernating ground squirrels, raises the intriguing possibility that the HIT-molecule in the peripheral circulation may be similar or identical in nature to their metabolic depressant, antabolone. There is a good possibility that the HIT molecule has its origin in the brain of hibernators and in fact may be a neurohormone secreted into circulation after appropriate central nervous system stimulation. After its secretion, it is bound to circulating albumin to protect it from the destructive action of non specific circulating proteases. Once bound or closely associated with albumin, the HIT molecule may influence isoenzyme and protein and lipoprotein synthesis which alters membrane composition and permeability to ion flux to such an extent that the tissues of the hibernator can now survive extended bouts of hypothermia without permanent damage.

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